REMARKS

The disclosure has been objected to for failure to follow the optional specification layout suggestions delimited in 37 C.F.R. §1.77(b). To properly effect the changes suggested by the Examiner Applicants submit herewith a *Second Amendment by Substitute Specification*, a *Second Substitute Specification Compliant with 37 C.F.R. §1.125(b)*, and a *Second Version With Markings to Show Changes Made*. To avoid Office confusion and to ensure that the *Second Substitute Specification Compliant with 37 C.F.R. §1.125(b)* is appropriately entered Applicants request that the Examiner write "Enter" or "OK to Enter" and the Examiner's initials in ink in the left margin of the first page of the *Second Substitute Specification*, as is directed by MPEP §608.01(q). Applicants further request that the Examiner acknowledge in the next Office communication that such entry has been appropriately effected.

Claims 1-3 have been rejected as being anticipated under 35 U.S.C. § 102(b), over U.S. Patent # 5,958,414; issued September 28, 1998; to Regnery *et al.* (hereinafter "Regnery"). Those rejections are hereby traversed.

I. The Claims are not Anticipated by Regnery: (a) Regnery does not disclose each and every element of the claims; (b) Regnery is non-enabling; and (c) Regnery does not contain the required *identity* of *structure*, *purpose*, and *result* of the instant claims.

A claim *rejection* for anticipation under 35 U.S.C. §102 requires that each and every element of the claimed invention be identically disclosed in a single prior art reference. Application of Marshall, 578 F.2d 301, 304 (Ct. of Cust. & Pat. App. 1978). *Accord* In re Paulson, 30 F.3d 1475 (Fed. Cir. 1994). In re Spada, 911 F.2d 705 (Fed. Cir. 1990). Ex parte Wilbanks, 2000 WL 33118609 (Bd. Pat. App. & Interf.). In infringement suits or DJ actions, wherein validity is at issue, this single reference / every element requirement is no less exacting.

Electro Medical Systems, S.A., v. Cooper Life Sciences, Inc., 34 F.3d 1048, 1052 (Fed. Cir. 1994)("Anticipation under 35 U.S.C. §102(b) requires the presence in a single prior art disclosure of each and every element of a claimed invention,..."). General Electric Company v. United States, 572 F.2d 745, 768 (Ct. of Claims 1978)("To anticipate a claim, a prior art reference must show each and every element claimed. Short of this, anticipation does not exist."). Lindemann Maschinenfabrik GmbH, v. American Hoist and Derrick Co., 730 F.2d 1452, 1458 (Fed. Cir. 1984)("Anticipation requires the presence in a single prior art reference disclosure of each and every element of the claimed invention, arranged as in the claim.").

The instant claims are directed to a polymer comprising a polyphosphazene backbone having pendant groups, at least a portion of which pendant groups bind to a receptor on human cells that activates innate immunity (independent claim 1). The claims are further directed to a similar polymer wherein the receptor activates an antigen specific Th1 immune response (independent claim 2), and still further directed to a composition that contains such a polymer and an antigen for the induction of antigen specific Th1 immune response (dependent claim 3).

The Examiner cites Regnery's col. 6, lines 32-64 for disclosing a polymer comprising a phosphazene backbone having pendant groups such as glucose, which, the Examiner argues, "inherently binds to a receptor on human cells that activates innate immunity including an antigen specific Th1 immune response." The Examiner further cites col. 6, lines 15-31 for disclosing a composition that contains such a polymer and an antigen for, the Examiner argues, "the induction of antigen specific Th1 immune response, specifically *B. heselae* antigen."

While Applicants appreciate the Examiner's *inherency* argument, that argument is based upon a reference wherein the glucose-substituted polyphosphazene is administered exclusively to cats and therefore has no contact with "a receptor on human cells"—a critical element of the

instant claims. The Examiner's attenuated argument—essentially being that the to-be-*cat-administered*, glucose-substituted polyphosphazene of Regnery *might* "bind to a receptor on human cells that activates innate immunity (claim 1), or *might* bind "to a receptor on human cells that activates an antigen specific Th1 immune response" (claim 2)—fails to properly vet the reference against the claims under the above-noted, and very exacting, *every element* rule. Instructively, a similarly attenuated, hyperbole-based anticipation rejection was reversed by the Federal Circuit's predecessor court in Application of Marshall, underscoring the stricture with which the *every element* rule is to be applied:

> "Rejections under 35 U.S.C. s 102 are proper only when the claimed subject matter is identically disclosed or described in the prior art. In re Arkley, 455 F.2d 586, 587, 59 CCPA 804, 807, 172 USPQ 524, 526 (1972). In other words, to constitute an anticipation, all material elements recited in a claim must be found in one unit of prior art...."
>
> "**Applying this rule of law to the present case, we must reverse the board's rejection of claims 1-4 under 35 U.S.C. s 102 since the primary reference, the PDR, does not disclose every material element of the claimed subject matter. These claims are directed to a weight control process. Applicant uses an effective amount of the anesthetic, oxethazaine, to inhibit release of the pancreatic secretory hormones, secretin and pancreozymin, in order to control weight. The PDR, however, teaches using drugs containing the anesthetic oxethazaine to inhibit release of the acid-stimulating hormone, gastrin, in order to treat esophagitis, gastritis, peptic ulcer and irritable colon syndrome. Nothing in the PDR remotely suggests taking oxethazaine to lose weight. If anyone ever lost weight by following the PDR teachings it was an unrecognized accident. An accidental or unwitting duplication of an invention cannot constitute an anticipation**. In re Felton, 484 F.2d 495, 500, 179 USPQ 295, 298 (Cust. & Pat.App.1973)." Application of Marshall, 578 F.2d 301, 304 (Ct. of Cust. & Pat. App. 1978)(emphasis added).

Similarly, while Regnery *does* disclose a polyphosphazene macromolecule containing myriad substitutents, among them glucose, Regnery does *not* state or suggest that this polyphosphazene macromolecule, glucose-substituted or otherwise, can activate innate immunity (as claimed), and much less an antigen specific Th1 immune response (as further claimed). In fact, nowhere in the *whole* of Regnery does the patent even mention *receptors* or *Th1 immune*

responses. And, Regnery's every mention of *human cells* is limited to their use as "[s]uitable eukaryotic cells on which to grow *B. henselae*" (col. 3, lines 38-45), which *B. henselae* by way of the Regnery *composition* is to be administered *exclusively* to the cat and *never* to the human, whereby the asserted-to-be *inherent* binding of the Regnery glucose-substituted polyphosphazene to *any* receptor on *any* human cell is an absolute impossibility! Accordingly, the single prior art reference, Regnery, fails to disclose each and every element of the claims, and therefore is not an anticipating reference.

In addition to disclosing a claim's every element, the single prior art reference on which the Examiner bases an anticipation rejection must be *enabling*, in that "the reference must describe the applicant's claimed invention sufficiently to have placed a person of ordinary skill in the field of the invention in possession of it." In re Spada, 911 F.2d 705, 708 (Fed. Cir. 1990). *Accord* In re Paulson, 30 F.3d 1474, 1479 (Fed. Cir. 1994). *See also* Akzo N.V., v. U.S International Trade Commission, 808 F.2d 1471, 1479 (Fed. Cir. 1986). The portions of Regnery on which the Examiner relies for *inherently* disclosing the instantly claimed polymers can hardly be said to be *enabling*, as those portions amount to little more than the disclosure of polyphosphazene containing an unlimited list of substituents. Indeed, Regnery concedes as much:

> "**The substituent ("R") can be any of a wide variety of moieties that can vary within the polymer, including but not limited to** aliphatic, aryl, aralkyl, alkaryl, carboxylic acid, heteroaromatic, carbohydrates, **including** glucose, heteroalkyl, halogen, (aliphatic)amino including alkylamino-, heteroaralkyl, di(aliphatic)amino- **including** dialkylamino-, arylamino-, diarylamino-, alkylarylamino-, -oxyaryl **including but not limited to** -oxyphenylCO_2H, -oxyphenylSO_3H, -oxyphenyl-hydroxyl and -oxyphenylPO_3H; -oxyaliphatic including -oxyalkyl, -oxy(aliphatic)CO_2H, -oxy(aliphatic)SO_3H, -oxy(aliphatic)PO_3H, and -oxy(aliphatic)hydroxyl, **including** oxy(alkyl)hydroxyl; -oxyalkaryl, -oxyaralkyl, -thioaryl, thioaliphatic **including** -thioalkyl, -thioalkaryl, thioaralkyl, --NHC(O)O-(aryl or aliphatic), --O--›(CH_2)xO]y--CH_2)--O--›(CH_2)xO]y(CH_2)xNH(CH_2)xSO_3H, and --O--(CH_2)xO]y-(aryl or aliphatic),

> **wherein x is 1-8 and y is an integer of 1 to 20**. The groups can be bonded to the phosphorous atom through, for example, an oxygen, sulfur, nitrogen, or carbon atom." (col. 6, lines 46-64, emphasis added).

As against the instantly claimed polymers and compositions the above-quoted Regnery selection clearly discloses little more than a prophetic laundry list of substituents, effectively putting nothing into the artisan's possession that could even approximate the claimed invention. Significantly, on the topic of *enablement* with regard to *anticipation* it has been said that:

> "**A mere naked formula for a chemical compound which teaches the art nothing about the product which it may represent, and does not put anyone in possession of the invention, is not the type of statement that should be relied upon for anticipation.** See In re Papesch, Cust. & Pat.App., 315 F.2d 381; Foremost Dairies, Inc. v. Watson, D.C., 132 F.Supp. 736; Mathieson Alkali Works, Inc. v. Coe, 69 App.D.C. 210, 99 F.2d 443; Cohn v. United States, 93 U.S. 366, 23 L.Ed. 907, and Rem-Cru Titanium, Inc. v. Watson, D.C., 147 F.Supp. 915." Phillips Petroleum Co. v. Ladd, 219 F.Supp. 366, 370 (U.S. Dist. Ct. D.C. 1963).

By extrapolation, the Regnery patent that the Examiner relies upon is even *less* enabling as an anticipating reference against the instantly-claimed polymers insofar as its aspirational laundry list of admittedly-*unlimited* substituents, giving rise to myriad prophetic compounds, is something considerably less *instructive* than the "mere naked formula," disclosed by the reference in Phillips Petroleum. Notably, the 1,3 butadiene polymer claims at issue in Phillips Petroleum were found *not* to have been anticipated by a reference *particularly* directed to the polymerization of 1,3 butadiene. 219 F.Supp. 366-368.

A similar laundry list reference that taught myriad-subsituted ethylene (-CN among the substituents) was also found to be non-enabling for anticipation purposes as against a claim directed to tetra**cyano**ethylene. (emphasis added). E.I. DuPont de Nemour and Co. v. Ladd, 328 F.2d 547 (D.C. Cir. 1964). Applying Application of LeGrice, the D.C. Circuit in DuPont noted the vagueries of the allegedly anticipating formula that was disclosed by the reference:

> " 'The proper test of a description in a publication as a bar to a patent as the clause is used in section 102(b) requires a determination of whether one skilled in the art to which the invention pertains could take the description of the invention in the printed publication and combine it with his own knowledge of the particular art and from this combination be put in possession of the invention on which a patent is sought. Unless this condition prevails, the description in the printed publication is inadequate as a statutory bar to patentability under section 102(b).' [quoting LeGrice]
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> **In the case before us, three of the four expert witnesses who testified regarding the anticipation of claims 1 and 3 by the earlier Alder patent (Theodore L. Cairns, Louis F. Fieser and Arthur C. Cope) stated that the general R(1), R(2), R3, R4 formula gives rise to an infinite number of possible compounds inasmuch as the acyl, alkyl, aryl, oxalkyl and esterified carboxylic acid groups mentioned in the formula represent classes or groups of substituents, within each of which are an infinite (or at least an indefinite) number of specific elements. Hence, even if one were to pick and hold constant a substituent for R1, R2 and R3 in the general formula, an infinite number of specific compounds would be suggested each time one of the above-named substituents was substituted for R4. The unequivocal testimony of these three witnesses was that, as a consequence of the vast scope of the general formula, this disclosure in the Alder patent would not suggest tetracyanoethylene to one skilled in the art of organic chemistry. As graphically stated by Dr. Cope:**
>
> > 'Taking all of the possible combinations and permutations of R1 and R2 and R3 and R4, and recognizing how many of them may be of infinite scope, **that formula is just about as broad as the universe; and, in my opinion, it is so broad that it would lead no chemist to the selection of any specific compound falling within that area.** * * * I would say that this is so broad that for a chemist to be led to any specific compound by this formula would be just about the same as being led to a specific *** baby being born at this moment.' "
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> E. I. DuPont de Nemours & Co. v. Ladd, 328 F.2d 547, 550-51 (D.C. Cir. 1964)(emphasis added).

Regarding *inherency* ("implicit disclosure") the DuPont court reconciled its finding that the tetracyanoethylene claims were novel, with an earlier C.C.P.A. decision, Baranauckas, on the basis that the reference at issue in DuPont (like the Regnery reference at issue here) was essentially a *theoretical list of possible compounds*, which Baranauckas noted to be outside the boundary of an appropriate anticipation reference:

> "It should be noted also that the court in Baranauckas stated
> '* * * though our decision is compelled by the existing law, we feel

> constrained to point out that there are limits to the doctrine of those cases. What the precise boundary lines are, we are unable now to discern. Certainly they do not extend so far as to permit publication of theoretical lists of hundreds or thousands of possible compounds to deny patent protection on such compounds to those who actually discovered them later. * * *' 228 F.2d at 416, 43 CCPA (Patents) at 731.
>
> **Even if there were some doubt that the Alder patent was not an implicit disclosure of tetracyanoethylene, within the meaning of the Baranauckas holding, the policy considerations suggested by that court would compel the same result. Certainly the Alder patent, allowing as it did an infinite number of possibilities, would be minimally described as an implicit 'publication of theoretical lists of hundreds or thousands of possible compounds,' and thus would not be an appropriate anticipation of a later patent application for a specific compound.**" E. I. DuPont de Nemours & Co. v. Ladd, 328 F.2d 547, 553 (D.C. Cir. 1964)(emphasis added).

By analogy, the Regnery patent is nothing more than a theoretical list of hundreds or thousands of possible compounds that may be derived from a polyphosphazene backbone, and thus would not be an appropriate anticipation of the instant polymer claims. Indeed, even the Examiner's description of the allegedly "*inherently*" anticipating, though at best inchoate, glucose-substituted Regnery polymer is ill-defined in the DuPont sense since Regnery notes that:

"The subsituent ("R") can be...*carbohydrates, including glucose,*"

Whereby the substituent on Regnery's inchoate, prophetic polymer might be a carbohydrate that *contains* a glucose molecule, and *not* a "pendant group such as glucose" as argued by the Examiner.

Even if, as argued by the Examiner, Regnery disclosed the instantly-claimed polymers, which it does not, the Examiner's inherency argument, based upon the allegedly glucose-substituted polyphosphazene disclosure, is tantamount to a rejection under the discredited Von Bramer doctrine, whereby a compound is *improperly* considered to be old owing to a reference's mere disclosure of a formula or sequence of letters. Citing the DuPont case for support the Court of Customs and Patent Appeals in Application of Brown held that the Von Bramer doctrine was

being misconstrued and that the proper test was whether the reference puts the subject matter in the possession of the public:

> "Apparently we are, in this case, in the field of what has come to be called the 'Von Bramer doctrine.' This doctrine, which appears to have resulted from In re Von Bramer et al., supra, seems over a period of years to have been tailored in some quarters to a principle which defeats the novelty of a chemical compound on the basis of a mere printed conception or a mere printed contemplation of a chemical 'compound' irrespective of the fact that the so-called 'compound' described in the reference is not in existence or that there is no process shown in the reference for preparing the compound, or that there is no process known to a person having ordinary skill in the relevant art for preparing the compound. **In other words, a mere formula or a mere sequence of letters which constitute the designation of a 'compound,' is considered adequate to show that a compound in an application before the Patent Office, which compound is designated by the same formula or the same sequence of letters, is old. We do not think that the Von Bramer case should be so construed.**" Application of Brown, 51 C.C.P.A. 1254, 1257 (Ct. Cust. & Pat. Apps. 1964)(emphasis added).
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> "**To the extent that anyone may draw an inference from the Von Bramer case that the mere printed conception or the mere printed contemplation which constitutes the designation of a 'compound' is sufficient to show that such a compound is old, regardless of whether the compound is involved in a 35 U.S.C. § 102 or 35 U.S.C. § 103 rejection, [FN5] we totally disagree. [FN6] Carrying such proposition to the extreme could, we think, result in a holding that a reference reciting the mere desirability of producing thousands of specifically named compounds without anything else is adequate to show that such compounds are old. We do not think that the Von Bramer case was intended to be carried to such an extreme.**
>
> **We think, rather, that the true test of any prior art relied on to show or suggest that a chemical compound is old, is whether the prior art is such as to place the disclosed 'compound' in the possession of the public.** In re LeGrice, 301 F.2d 929, 49 CCPA 1124; E. I. DuPont de Nemours & Co. v. Ladd, 328 F.2d 547, (D.C.Cir. 1964).
>
> Applying the above test to the facts before us, we do not think that Clark's reference to his unsuccessful attempts to prepare fluorine-containing silicone homopolymers would place such homopolymers in the possession of the public and as a corollary would place appellant's invention in possession of the public. [FN7] As Robinson [FN8] in 1890 said in section 330 entitled 'Prior Publication: Publication must Fully Communicate the Invention to the Public':
>
> > 'Finally, the description must place the invention in the possession of the public as fully as if the art or instrument itself had been practically and publicly employed. In order to accomplish this, it must be so particular and definite that from it alone, without

> experiment or the exertion of his own inventive skill, any person versed in the art to which it appertains could construct and use it.'
> **Certainly it cannot be said that the disclosure in the Clark reference is such that it would, without the exertion of another's own 'inventive' skill, place appellant's invention in the possession of the public.**" Application of Brown, 51 C.C.P.A. 1254, 1259 (Ct. Cust. & Pat. Apps. 1964)(emphasis added).

As was noted above, Regnery does *not* state or suggest that a polyphosphazene macromolecule, glucose-substituted or otherwise, can activate innate immunity (as claimed), or an antigen specific Th1 immune response (as further claimed). When it is considered that Regnery is silent with regard to *receptors*, *Th1 immune responses*, or *binding to human cells*, and further considered that the Regnery composition is to be administered *exclusively* to the cat and *never* to the human, it must be appreciated that Regnery does not place the invention in the possession of the public *fully*--as if it "had been practically and publicly employed." In order to arrive at the instant claims the artisan could *not* rely exclusively on Regnery without experiment or exertion. Rather the artisan would have to resort to his own inventive skills—such as formulating a *definite* polyphosphazene polymer with substituent pendant *groups specifically capable of binding to a receptor on human cells that activates innate immunity*, and then administering such formulation to a *human* patient or subject.

More recently the *public possession / enablement* focus has been considered in the context of a D.J. action wherein the *Federal Circuit* rejected an inherency-based anticipation argument that was founded upon the possibility of there existing a *given set of circumstances* that would provide the inherency:

> "At trial, EMS challenged the validity of claim 20 of the '402 patent on the basis that it was anticipated by U.S. Patent 2,405,854 to Ruemelin under 35 U.S.C. § 102(b). Anticipation must be proved by clear and convincing evidence. Verdegaal Bros., Inc. v. Union Oil Co., 814 F.2d 628, 632, 2 USPQ2d 1051, 1053 (Fed.Cir.), cert. denied, 484 U.S. 827, 108 S.Ct. 95, 98 L.Ed.2d 56 (1987). Anticipation under 35 U.S.C. § 102(b) requires the presence in a single prior art disclosure of each and every element of a claimed invention, Lewmar Marine,

> Inc. v. Barient, Inc., 827 F.2d 744, 747, 3 USPQ2d 1766, 1767 (Fed.Cir.1987), cert. denied, 484 U.S. 1007, 108 S.Ct. 702, 98 L.Ed.2d 653 (1988), and is a question of fact subject to review under the clearly erroneous standard, Ralston Purina Co. v. Far-Mar-Co., Inc., 772 F.2d 1570, 1574, 227 USPQ 177, 179 (Fed.Cir.1985).
>
> The court determined that EMS had failed to introduce clear and convincing evidence that the Ruemelin patent discloses every element of claim 20. Specifically, the court found that the Ruemelin patent did not disclose a substantially unpressurized flow of liquid or a continuous liquid curtain surrounding the pressurized jet of particle-laden gas. Electro I, slip op. at 55. **EMS asserts that these features are "inherent" in the Ruemelin patent because, although Ruemelin discloses a blasting and spraying gun utilizing pressurized liquid, the Ruemelin device "could be set to any water pressures."**
>
> **We do not agree that the subject matter of the claim was anticipated. "The mere fact that a certain thing may result from a given set of circumstances is insufficient to prove anticipation."** Continental Can Co. v. Monsanto Co., 948 F.2d 1264, 1268-69, 20 USPQ2d 1746, 1749 (Fed.Cir.1991) (quoting In re Oelrich, 666 F.2d 578, 581, 212 USPQ 323, 326 (CCPA1981)) (emphasis added). **EMS was required to prove that an unpressurized flow is necessarily present in the Ruemelin disclosure, and that it would be so recognized by persons of ordinary skill.** Id. at 1268, 20 USPQ2d at 1749. **EMS did not discharge its burden; thus, the district court properly concluded that EMS failed to prove invalidity of claim 20.**" Electro Medical Systems, S.A. v. Cooper Life Sciences, Inc., 34 F.3d 1048, 1052 (Fed. Cir. 1994)(emphasis added).

Similarly, the Examiner has failed to cite any column and line of Regnery that would impart to one of ordinary skill in the art the knowledge that a polyphosphazene polymer (or composition containing such polyphosphazene polymer) as claimed would *bind to a receptor on human cells that activates innate immunity*, or would *bind to a receptor on human cells that activates an antigen specific Th1 immune response.* In the decidedly clear absence of that teaching the *inherency* argument is tantamount to nothing more than conjecture as to what may result under a given set of circumstances—none of which circumstances are disclosed by Regnery, in view of the fact that his composition is to be administered exclusively to the cat. The artisan (or public) therefore is not put in possession of the claimed invention.

Not only is Regnery bereft of this necessary teaching, but, significantly, *other* prior art

references (that are hereto-attached) contradict the foundation on which the Examiner's *inherency* argument is based. While it is true that some macromolecules containing sugar moieties—such as glucans containing glucose moieties; mannans; and chitin—can activate the immune system, still other macromolecules containing sugar moieties—such as lipophosphoglycan containing glucose moieties—do *not* activate the immune system, but rather, *inhibit* proinflammatory responses in macrophages. (*See* Aderem *et al.*; *Toll-like receptors in the induction of the innate immune response*, Nature, Vol. 406, P. 783, August 2000; attached hereto as **Exhibit A**). Yet, *still other* references teach that the relationship between the structures of glucans and their stimulatory activities are open to debate. (*See* Kataoka *et al.*; *Activation of Macrophages by Linear (1→3)-ß-D-Glucans*, Journal of Biological Chemistry, 2002, Vol. 277, No. 39, page 36825-36826; attached hereto as **Exhibit B**). Accordingly, the Examiner's rejection of the claims as being *inherently* anticipated is further traversed, both because Regnery is non-enabling and, as is shown by prior art Exhibits A & B, the ability of polyphosphazenes to activate the innate immune system cannot be anticipated based merely on the fact that the polyphosphazene may contain a sugar moiety.

In addition to the *every element* rule and the *enablement* requirement discussed above, the allegedly anticipating reference's *every element* "must function in substantially the same way to produce substantially the same result." Tate Engineering, Inc. v. United States, 477 F.2d 1336, 1342 (U.S. Ct. of Claims 1973). In other words, that single prior art reference is *not* anticipating if it does not disclose an "identity of *structure*, *purpose* and *result*" with the claimed invention. Id. (emphasis added). *See also* Straussler v. United States, 339 F.2d 670, 671 (U.S. Ct. of Claims 1964).

On this point, the Examiner's *inherency* argument is further undermined by still *more*

prior art references that suggest that the response described in Regnery is actually antibody based, and *not* Th1 based as instantly-claimed. Polyphosphazene adjuvants, such as PCPP, have been widely studied and have been determined to induce only antibody based Th2 immune responses. *See each of* (Chen *et al.*, *Targeting epidermal Langerhans cells by epidermal powder immunization*; Cell Research (2002);12 (2):97-104; attached hereto as **Exhibit C**); (Payne *et al.* (1995) Chapter 20,473-493 in Vaccine Design: *The Subunit and Adjuvant Approach*; ed. Powell and Newman; Plenum Press, NY, 1995; attached hereto as **Exhibit D**); (Lu *et al*, *Utility of SHIV for Testing HIV-1 Vaccine Candidates in Macaques*; J Acquir Immune Defic. Syndr. (1996),12,99-106; attached hereto as **Exhibit E**) and (Payne *et al.*; *Poly[di(carboxylatophenoxy)phosphazene](PCPP) is a potent immunoadjuvant for an influenza vaccine*, Vaccine (1998), Vol. 16, No. 1., pp.92-98; attached hereto as **Exhibit F**). Of course, these data do not imply that the *B. henselae* antigen is lacking in sequences that would induce a Th1 response, but rather that PCPP has only demonstrated the capacity to stimulate Th2 responses to antigen specific sequences.

While the Regnery formulation of *B henselae* antigen with PCPP induced protective immunity in domestic cats from challenge with *B. henselae*, it must be considered that a prior art reference by Karem *et al.* (Regnery himself among the *et al.*) teaches that in the closely related *B. bacilliformis* long term protective immunity may be conferred by antibody, supported by the induction of neutralizing antibodies to outer membrane proteins of the bacteria. (See Karem *et al.*; *Bartonella henselae, B. quintana, and B. bacilliformis: historical pathogens of emerging significance*; Microbes and Infection (2000) 2, 1193-1205; attached hereto as **Exhibit G**). Furthermore this correlation of protective immunity in cats with antibodies was strengthened by the recent demonstration of passive antibody transfer of feline anti-Bartonella serum protected

cats from clinical disease on challenge with *B henselae*. (See O'Reilly *et al.*; *Passive Antibody to Bartonella henselae Protects against Clinical Disease following Homologous Challenge but Does Not Prevent Bacteremia in Cats*; Infection and Immunity (2001), 69,3, 1880-1882; attached hereto as **Exhibit H**). This data indicates that the basis of *B henselae* immunity in domestic cats is dependent on an antibody based Th2 response, and not Th1 as claimed. Accordingly, the single prior art reference, Regnery, fails to disclose an identity of structure, purpose, and result, as any *inherent* teachings it might contain vis-à-vis the instant claims are *expressly* undermined by the hereto-attached rebuttal art references for each of the above-noted the reasons. Therefore, the instant claims are not anticipated by Regnery.

Lastly, the Examinr must note that the fact that new dependent claims 4-6 respectively further limit original claims 1-3 by adding the negative limitation that the pendant groups do not include glucose. The Examiner's attention is directed to MPEP §2173.05(i) for the proposition that: "If alternative elements are positively recited in the specification they may be explicitly excluded in the claims." Applicants would further note for the Examiner that glucose as a pendant group is positively recited in the specification as an alternative element, and therefore may be explicitly disclaimed:

> "In one aspect of the present invention, wherein the polyphosphazene contains pendant or side groups wherein at least a portion thereof binds to a mannose receptor on immune cells, such pendant group may be a saccharide which may be a monosaccharide or an oligosaccharide or a polysaccharide. In a preferred embodiment, such saccharide (in the form of a monosaccharide or terminal saccharide of an oligosaccharide) is one which contains mannose, fucose, N-actylglucosamine **or glucose**. Such monosaccharide or terminal saccharide of an oligosaccharide may be, for example, mannose, **glucose**, GlcNAc, fucose, galactose, GalNAc, and mannose, but it is to be understood that the present invention is not limited to such preferred materials." (See page 3 of the herewith-included *Second Substitute Specification*)(emphasis added).

In view of the foregoing, Applicants submit that neither independent claim 1 nor

independent claim 2 is anticipated by Regnery. The remaining claims, all of which are dependent claims are therefore similarly not anticipated by Regnery.

In further view of the foregoing, Applicants submit that the application is in condition for allowance, and they therefore request its prompt passage to issue.

Applicants herewith-include a check for $150 to cover the cost of six claims in excess of 20, at the small entity level. It is believed that no further fees are due. However, if any fee is due it should be charged to Deposit Account No.: 03-0678. Similarly, any credit for overpayment should be charged to Deposit Account No.: 03-0678.

CERTIFICATE OF MAILING

Deposit Date: January 12, 2005

I hereby certify that this paper and the attachments hereto are being deposited today with the U.S. Postal Service with sufficient postage as First Class Mail to Addressee, under 37 CFR 1.8, on the date indicated above addressed to:

Commissioner of Patents
P.O. Box 1450
Alexandria, VA 22313-1450

1/12/05

Raymond E. Stauffer, Esq. **Date**

#243239 v1 - Response to Non-Final Rejection

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Toll-like receptors in the induction of the innate immune response

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The innate immune response is the first line of defence against infectious disease. The principal challenge for the host is to detect the pathogen and mount a rapid defensive response. A group of proteins that comprise the Toll or Toll-like family of receptors perform this role in vertebrate and invertebrate organisms. This reflects a remarkable conservation of function and it is therefore not surprising that studies of the mechanism by which they act has revealed new and important insights into host defence.

The immune response to microbial pathogens relies on both innate and adaptive components[1]. The immediate, innate response is mediated largely by white blood cells such as neutrophils and macrophages, cells that phagocytose and kill the pathogens, and that concurrently coordinate additional host responses by synthesizing a wide range of inflammatory mediators and cytokines[2]. In macrophages, the infectious agent is killed and degraded within the maturing phagosome, and components of the pathogen are presented to T cells, resulting in the activation of the adaptive immune response and the establishment of protective immunity[2]. A primary challenge to the innate immune system is the discrimination of a large number of potential pathogens from self, with the use of a restricted number of receptors. This problem is compounded by the tendency of pathogens to mutate. This challenge has been met by the evolution of a variety of receptors that recognize conserved motifs on pathogens that are not found in higher eukaryotes. These motifs have essential roles in the biology of the invading agents, and are therefore not subject to high mutation rates. Janeway & Medzhitov[3] have provided a set of definitions to formalize a description of the components of the innate immune system. They propose calling the motifs pathogen-associated molecular patterns (PAMPs), and their cognate binding partners on the phagocytes pattern-recognition receptors[3]. Pathogen-associated motifs include mannans in the yeast cell wall, formylated peptides and various bacterial cell-wall components such as lipopolysaccharide (LPS), lipopeptides, peptidoglycans and teichoic acids[3] (Table 1). There are two principal classes of pattern recognition receptor: those that mediate phagocytosis and those that lead to the activation of pro-inflammatory pathways[2,3].

The best studied model of innate immunity is one using Gram-negative bacteria and bacterial endotoxin (LPS)[4]. LPS is considered to play a key role in the septic shock syndrome in humans owing to its profound effects on the innate immune system. It is therefore not surprising that studies of the pathogenesis of septic shock has time and again provided the key insights into the molecular basis of innate immunity. We discuss many of these recent advances below.

LPS is the prototypic activator of innate immunity; it is active at concentrations below 1 nM, and studies with LPS have revealed the sequence and mechanisms of the essential responses[4]. Despite this focus on LPS, it is now clear that other microbial products, structurally distinct from LPS, act as potent activators of the innate immune system. It is therefore useful to consider briefly the various classes of these activating substances, and then discuss them in view of what is known at the mechanistic level. But although it has been useful to analyse the pathways leading to immune activation triggered by purified microbial components, it is important to remember that the immune system encounters a 'cocktail' of these molecules when interacting with pathogens *in vivo*.

Components of pathogens that activate immunity

Within the known phyla of bacteria, there are four groups of especially significant human pathogens. These are Gram-negative and Gram-positive bacteria, mycobacteria and spirochaetes. A wide variety of bacterial components are capable of stimulating innate immune responses. These include LPS, peptidoglycan, lipoteichoic acid, lipoarabinomannan (LAM), lipopeptides and bacterial DNA (see Table 1). LPS is the principal component of Gram-negative bacteria that activates the innate immune system. The chemical structure of these complex outer-membrane glycolipids has been studied extensively and discussed in numerous reviews[5,6]. The essential structural feature of LPS that governs interactions with the innate immune system is known as lipid A (ref. 6). Regardless of the type of Gram-negative bacterium, the lipid A is composed of a diglucosamine backbone containing ester-linked and amide-linked long-chain fatty acids. Recent results suggest that the fatty-acid composition might dictate the binding specificities of key receptors[7,8]. Bacterial lipoproteins are also potent stimulators of inflammatory responses, and are found in all Eubacteria[9]. For example, lipoproteins from mycobacteria, mycoplasma and spirochaetes activate macrophages. These outer-membrane lipoproteins contain a lipid-modified cysteine residue at the amino terminus; this lipid-containing domain is essential for the biological effects of the lipoproteins[9]. Gram-positive bacteria contain cell walls composed of a number of potentially biologically active molecules, including peptidoglycan, lipoteichoic acid and lipopeptides[9]. LAM is a key stimulator of innate immunity found in mycobacteria; it and a variety of peptidoglycan and lipopeptide species are the molecules that endow complete Freund's adjuvant with its efficacy[10]. Among the spirochaetes only *Leptospira* have LPS in the outer membrane[11]. Although leptospiral LPS might be an

important component in this group of bacteria, current opinion supports the contention that outer-membrane lipoproteins are the principal activating signal to the innate immune system during infection by spirochaetes[12].

For other microbial pathogens such as yeast or fungi and protozoan parasites, the spectrum of molecules known to stimulate innate immune responses are further extended. For example, in *Candida albicans* cell-wall constituents such as glucan, chitin, mannans and mannoproteins are potent immune modulators[13], whereas in *Plasmodium falciparum* glycosylphosphatidylinositol molecules are stimulatory[14]. Interestingly, not all cell-wall constituents activate innate immune responses; lipophosphoglycan in *Leishmania donovani* inhibits proinflammatory responses in macrophages[15]. It will be important to continue to characterize the structure of additional molecules present in the outer membranes and/or cell walls of pathogens that activate the innate immune system. As discussed below, there are at least nine members of the Toll family and only for two of them have ligands been defined, suggesting that many ligand–receptor pairs remain to be identified.

Recognition of molecular motifs on pathogens

Studies of the pathogenesis of Gram-negative septic shock led to a new level of understanding of mechanisms of innate immunity[16]. First came the discovery and characterization of LPS-binding protein (LBP); subsequently the identification of the importance of CD14 resulted in studies that formed the framework for our current state of knowledge[17,18]. Extensive biochemical, genetic and animal-model data support the concept that activation of host defence mechanisms when Gram-negative bacteria are present requires the recognition of LPS by mechanisms dependent on LBP and CD14 (ref. 16) (Fig. 1). Thus, LBP is an opsonin and CD14 is an opsonic receptor for complexes of LPS (or LPS-containing particles such as bacteria) and LBP. CD14 has been implicated in cell activation processes involving other products of microbial pathogens including LAMs, peptidoglycans and outer-membrane lipoproteins[16]. The question of the involvement of opsonic proteins for these varied stimuli has not been answered and remains to be addressed in detail. Moreover, there are few, if any, relevant details about the structure of CD14 that provide insight into the ligand-binding pocket of this protein. The fact that both CD14 and members of the Toll family contain multiple leucine-rich repeats suggests novel ligand-binding sites as well as protein–protein interactions at the cell surface that are not yet understood. Numerous studies support the contention that CD14 functions solely as a ligand-binding protein (LPS or LPS–LBP complexes) that does not participate in the generation of a transmembrane signal. Rather, many workers hypothesized that one or more additional transmembrane proteins acted in concert with LPS–CD14 complexes to initiate the signalling processes leading to cell activation. Extensive efforts to identify this putative transmembrane protein were unsuccessful until very recently.

Approximately 8 years after the initial publications delineating the importance of LBP and CD14 came the next main advance in understanding innate immunity: the identification of the putative transmembrane protein that acted with CD14 to generate a transmembrane signal linked to LPS-induced cell activation. The impetus for this advance was two seminal discoveries: the role of the Toll-like receptors (TLRs) in innate immunity in *Drosophila*[19,20], and the identification of a TLR homologue as the gene responsible for LPS responses in two natural mouse mutants[21–23]. These results marked the beginning of what will most certainly be a large leap forward in understanding how innate immune responses function in regulating responses to infection with microbial pathogens. They are of equal importance to the discoveries underlying our understanding of plasma-membrane receptors that control adaptive immune responses.

The TLR family

Although *Drosophila* has no adaptive immune system, it is very resistant to microbial infections[24]. This is because the innate immune

Table 1 PAMPs and pattern recognition receptors

PAMP	Pathogen(s)	Essential ligand	Pattern recognition receptors	Biological sequelae
LPS	Most Gram-negative bacteria	Lipid A	LBP, CD14	Enhance inflammatory response mediated by TLRs
			TLR4, TLR2*	Recognize LPS and initiate inflammatory response
			Scavenger receptor	Endocytosis of LPS (non-inflammatory), ?phagocytosis
Lipoproteins	Eubacteria	Amino-terminal tripalmitylated cysteine† generated by the signal peptidase II cleavage system	TLR2	Initiates inflammatory response
Peptidoglycan	Most bacteria	Undefined	CD14	Enhances inflammatory response
			TLR2	Initiates inflammatory response
Lipoteichoic acid	Many Gram-positive bacteria	Undefined	TLR2, TLR4‡	Initiates inflammatory response
CpG	Many microbial pathogens	Unmethylated CpG-containing oligonucleotide§	Undefined	Initiates inflammatory response
Lipoarabinomannan	Mycobacteria	Undefined	TLR2	Initiates inflammatory response
			CD1	Presents glycolipid to αβ T cells
N-formyl-Met	Prokaryotes	Amino-terminal *N*-formylmethionine of proteins synthesized *de novo*	f-Met receptors 1 and 2	Chemotaxis and release of inflammatory mediators (?)
Mannans and mannoproteins	Yeast	Undefined	Mannose receptor	Phagocytosis, endocytosis and initiation of inflammatory response
			Mannose-binding protein	Opsonization and complement fixation
Zymosan (yeast cell wall)	Yeast	Undefined	Mannose and β-glucan receptors	Phagocytosis
			TLR2	Initiates inflammatory response
Heat shock proteins	Prokaryotes and eukaryotes	Undefined	Undefined‖	Initiate inflammatory response and promote T cell dependent immune responses

* TLR2's role as an LPS receptor is based on studies *in vitro*, and, although TLR2 may function as a physiological receptor for LPS, evidence to support this function *in vivo* has not yet been demonstrated.
† Mycoplasma lipoproteins have been shown to have a dipalmitylated amino-terminal cysteine residue, which is also recognized by TLR2. These findings indicate that not all three fatty acid ester-linked chains are necessary for recognition.
‡ LTA is recognized by TLR2 in systems *in vitro*, and by TLR4 in studies with knockout mice. These differences have not been reconciled.
§ Flanking sequences are also influential.
‖ Studies with Hsp60 family members have shown that C3H/HeJ mice are defective in inflammatory signalling in response to these proteins, implicating TLR4 as a pattern recognition receptor for foreign and endogenous Hsp60 family gene products.

system provides protection that is in part due to the synthesis of potent antimicrobial peptides. These peptides are induced in response to signalling pathways activated by at least two members of the TLR family found in *Drosophila*, dToll and 18-wheeler. The activation of dToll induces an anti-fungal peptide, drosomycin, whereas the ligation of 18-wheeler induces an antibacterial peptide, attacin[19,20,25]. An essential step in the activation of such responses in *Drosophila* seems to be the activation of a proteolytic cascade that produces peptidic ligands for the Toll family of receptors. Whether or not this mechanism is unique to *Drosophila* or has been conserved in mammalian cells remains unanswered. Remarkably, these studies suggest that the TLRs were capable of discriminating between fungi and bacteria, and further that they were capable of inducing an appropriate and distinct antimicrobial response. Activation of these pathways in *Drosophila* via the membrane receptor initiates an intracellular kinase cascade that ultimately produces a translocation of transcription factors, Dif and Relish[24], from cytoplasm to nucleus. Dif and Relish are homologous to NF-κB, a transcription factor known to activate a variety of inflammatory mediators and cytokines including tumour necrosis factor-α (TNF-α) and interleukin-12 (IL-12)[25], but it is still not clear what differences in signalling downstream of dToll and 18-wheeler account for the divergent patterns of gene expression. Because is likely that mammalian cell activation via different TLRs produces distinct patterns of gene expression, the continued study of innate immunity in *Drosophila* is likely to provide new clues about related processes in mammalian cells.

Janeway and colleagues, in the search for receptors within the innate immune system, began a search for dToll-related proteins. Their efforts provided the next key advance in our understanding of innate immunity: they identified the first human homologue of *Drosophila* toll, initially termed human Toll and subsequently termed TLR4 (ref. 26). This observation suggested a probable link to innate immunity in general because a constitutively active mutant of TLR4 activated NF-κB-controlled genes such as IL-1, IL-6 and IL-8 (ref. 26). Importantly, constitutively active TLR4 also induced members of the B7 family, molecules that are required for the activation of naive T cells by antigen-presenting cells[26]. TLR4 was therefore a potentially important link between pathogen detection and the induction of the adaptive immune response. It was reasonable to wonder whether other members of this family were present in mammalian cells; soon afterwards, four more human TLRs (hTLRs) were discovered. Subsequent studies by others provided a detailed analysis of the structural features that link these proteins to *Drosophila* toll and to the IL-1 receptor family[27]. Importantly, Rock *et al.* highlighted the general structural features of the TLR family, namely the presence of multiple leucine-rich repeats in the ectodomain and the Toll-homology domain found in the cytoplasmic tail of all members of this protein family.

Establishing a link between TLRs and pathogen recognition by means of CD14-dependent mechanisms was essential to advance our understanding of the possible role of TLRs in innate immunity. Progress came rapidly as several groups established model systems for defining the function of one TLR, TLR2. First, it was demonstrated that the ectopic expression of TLR2 would support the LPS-induced activation of NF-κB in the transfected cells[28,29]. Second, these model systems permitted an analysis of downstream signalling pathways. Importantly, both groups provided data to support the contention that TLR2 signalling to NF-κB involves steps that are similar to those used by the IL-1 receptor[28,29]. These common steps include the involvement of the adaptor protein MyD88, the serine kinase IL-1R-associated kinase (IRAK) and another adaptor protein, TBF-receptor-associated factor 6 (TRAF6). It must be kept in mind that these initial reports did not document a physiological role for TLR2 as an LPS receptor. In fact the function of TLR2 as an LPS receptor remains unclear. As noted below, genetic data strongly support the contention that TLR4 is the predominant, if not the exclusive, receptor for LPS isolated from most Gram-negative organisms.

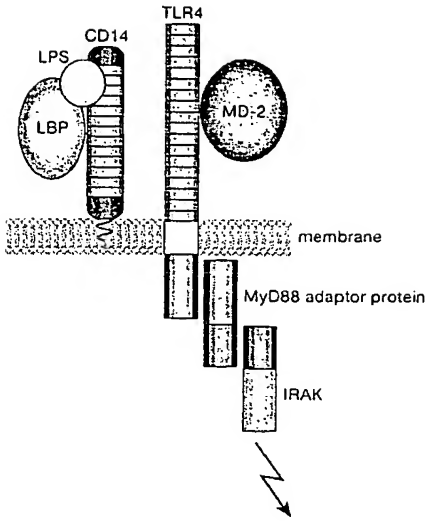

Figure 1 Recognition of LPS on the surface of phagocytes. LPS is opsonized by LBP, and the complex is recognized by the opsonic receptor, CD14, on the macrophage surface. CD14 associates with the cell surface by means of a glycolipid linkage and is not capable of generating a transmembrane signal. It is likely that the LPS–LBP–CD14 ternary complex activates TLR4 in some way, which in turn signals through the adaptor protein MyD88 and the serine kinase IRAK. The mechanism by which TLR4 is activated is not known and could be either direct or indirect. Both TLR4 and CD14 contain multiple leucine-rich repeats (brown); and these domains might facilitate protein–protein interaction. MD-2 is a secreted protein that binds to the extracellular domain of TLR4 and is important in its signalling.

Again, the many efforts to understand the pathogenesis of septic shock paved the way to our current understanding of how LPS is recognized in physiological settings. For more than two decades it had been understood that certain strains of mice were resistant to LPS and that this resistance was a consequence of a genetic defect. The seminal work of Beutler and co-workers demonstrating that the genetic defect in two strains of mice that are hypo-responsive or non-responsive to LPS was linked to TLR4 (refs 21, 22) led to a firm conclusion about how LPS is recognized. The co-dominant *Lpsd* allele of the C3H/HeJ strain was a result of a mis-sense mutation in the third exon of TLR4; this mutation was predicted to result in a Pro 712→His substitution[21,22]. When this mutation was introduced into wild-type TLR4, the receptor was converted into a dominant-negative mutant that inhibited LPS-dependent responses in a transfected macrophage cell line[30]. Another LPS-resistant strain, C57Bl/10ScCr, was shown to be homozygous for a null mutation of TLR4 (refs 21, 22). These findings provided the first direct link between the TLRs and physiological responses to LPS. This contention was supported by the demonstration that TLR4-null mice had a phenotype that was similar to that of the C3H/HeJ strain[23]. Another important finding supporting an essential role for TLR4 in LPS-dependent responses was provided by the observation that Chinese hamsters respond normally to LPS even though they carry a null allele for TLR2 (ref. 31). Finally it was shown that a dominant-negative mutant of TLR2 did not effect LPS responsiveness in transfected macrophages[30]. Thus a large gap in our knowledge about how LPS is recognized was closed by the combined efforts of many investigators with a primary interest in defining the mechanisms responsible for septic shock.

Receptors of the acquired immune system are composed of multi-protein complexes that permit the tight regulation of cellular activation through a variety of mechanisms. It is therefore not

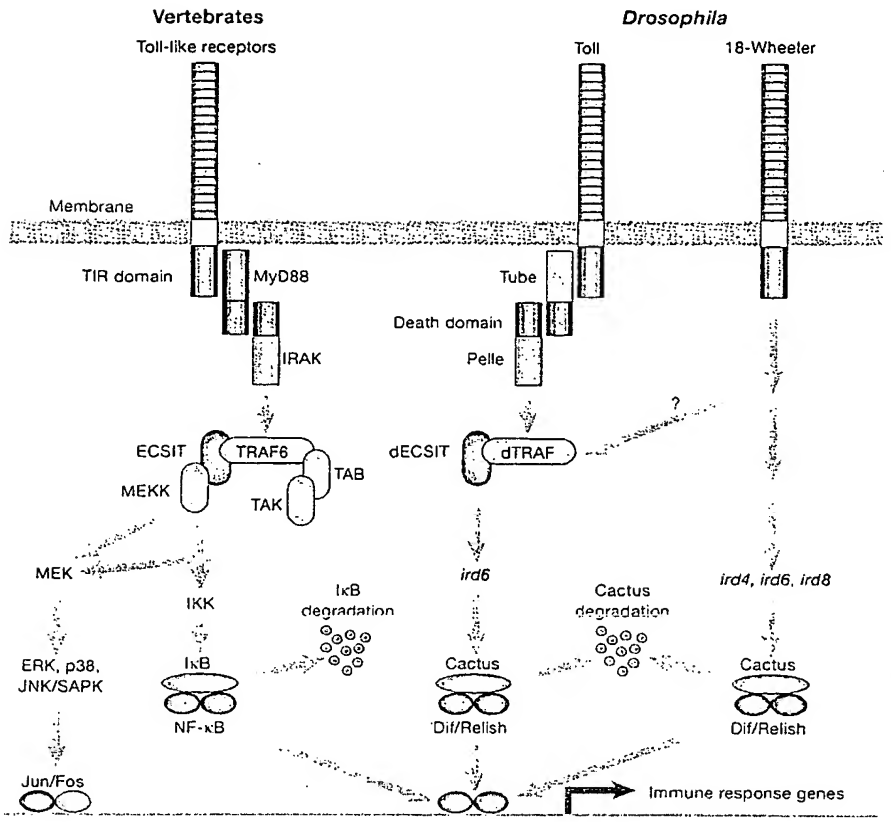

Figure 2 Signalling pathways activated by TLRs in vertebrates and in *Drosophila*. The TLRs have an intracellular domain that is homologous with that of the IL-1 receptor, and is known as TIR. TIR binds to a homologous domain in an adaptor protein, MyD88, which also contains a death domain; this interacts with a death domain in the serine kinase IRAK. IRAK interacts with an adaptor known as TRAF6. TRAF6 links to the MAP 3-kinase TAK-1, through an adaptor TAB2. TAK-1 is involved in the activation of the transcription factor NF-κB through the activation of IκB kinases, and in the activation of the AP-1 transcription family members Jun and Fos, by way of additional MAP kinases. Both AP-1 transcription family members and NF-κB are required for the transcription of immune response genes. TRAF6 is known to act through more than one pathway. For example, the adaptor ECSIT (evolutionarily conserved intermediate in Toll pathways) bridges TRAF6 to the MAP 3-kinase MEKK-1. The *Drosophila* Toll pathway is similar to that of the vertebrate TLRs. Toll links to an adaptor tube, the functional homologue of MyD88. Tube binds the kinase Pelle, a homologue of IRAK. A number of other homologues of the vertebrate pathways are found in *Drosophila*, including dTRAF and dECSIT, although it is not yet clear where precisely they fit in to the signalling pathway. Similarly, additional *Drosophila* genes, including *ird4*, *ird6* and *ird8*, have been have been found through genetic screens, although precisely how they fit into the toll signalling pathway is not yet known. Finally, Cactus is a homologue of IκB, whereas Dif and Relish are homologues of NF-κB. See the text for a more complete description of the signalling pathway.

surprising that receptors that regulate innate immune responses might have similar degrees of complexity. This likelihood is best illustrated by the current level of understanding of the LPS receptor. The importance of CD14 and TLR4 is well established. Most recently an additional protein, termed MD-2, has been shown to be important for signalling via TLR4 (ref. 32). MD-2 is a secreted protein that apparently functions by binding to the extracellular domain of TLR4, where it facilitates LPS responsiveness, perhaps by stabilizing TLR4 dimers. However, the precise function of MD-2 in the LPS receptor complex is unknown at present. Defining the function of MD-2 in terms of its role in supporting LPS signalling as well as determining the interrelations of the proteins of the LPS receptor remain as important questions for future research.

The importance of TLR2 as an LPS receptor still remains unresolved, although the genetic data strongly support the contention that TLR4-dependent signalling mechanisms predominate. Is there a species difference? The results from studies *in vitro* implicating TLR2 in LPS signalling were obtained with the human receptor, whereas the genetic studies were performed in the mouse. This latter argument seems unlikely in view of a very recent report that an antibody against TLR2 does not inhibit LPS-induced TNF-α production in human monocytes, although it inhibits TLR2 activation by heat-killed *Listeria monocytogenes*[33]. It seems likely that TLR2 can function as an LPS receptor when overexpressed but that it is not important for LPS responses *in vivo*. It remains to be determined whether or not TLR2 functions as a receptor for LPS from other organisms, such as spirochaetes or from other bacteria with lipid A of atypical structure. In contrast, a substantial number of reports detailing biochemical and genetic data provide strong support for the contention that TLR2, but not TLR4, is a receptor for components of Gram-positive bacteria, mycobacteria, yeast and other microbial pathogens. Thus, TLR2-null mice and TLR2-null Chinese hamster ovary cells respond to LPS but not to Gram-positive bacterial component[34,35]. Similarly, macrophages expressing a dominant-negative mutant of TLR2 do not respond to Gram-positive bacteria but are fully functional when challenged with LPS[30]. More or less simultaneously, several groups provided evidence that Gram-positive cell-wall components, including peptidoglycan and lipoteichoic acid, activate cells via TLR2, and that TLR2 signals in response to lipopeptides from a wide variety of bacteria[34–37]. However, whereas TLR4 seems to be selective for LPS (although there is an example of its signalling the response to HSP60 (ref. 38)), TLR2 is much more promiscuous. Not only does TLR2 signal in response to different chemical structures such as peptidoglycan and lipopeptide[35–37], but it is also required for pro-inflammatory signalling to mycobacterial cell-wall components including lipopeptides, lipoarabinomannan and mycolylarabinogalactan–peptidoglycan complexes[39–42]. In addition, TLR2 is required for the pro-inflammatory pathways stimulated in macrophages by zymosan, a yeast cell-wall preparation[30]. Taken together, these results support the contention that TLR4 functions as a receptor for LPS from Gram-negative bacteria and TLR2 is involved in the recognition of multiple products of Gram-positive organisms, mycobacteria and yeast. This ability of TLR4 and TLR2 to discriminate between pathogens is remarkably similar to the selectivity between pathogens observed for the *Drosophila* TLRs, dToll and 18-wheeler[19,20] described above. One important question that has yet to be answered is the sequence of events that occur after TLR2 or TLR4 is engaged by a physiological ligand. This includes downstream signalling molecules and perhaps

most importantly a description of the genes that are induced as a result of activation of these receptors.

So far, nine different mammalian TLRs have been described[27,43,44] and the complete sequence of TLR10 has been elucidated (T.-H. Chuang and R.J.U., unpublished observations). It is not unreasonable to assume that additional TLRs might be found as genomic databases become more complete. So far, genetic data suggest that the TLRs have unique functions and are not redundant. The identification of ligands for the TLRs is therefore an important area of research. It is safe to assume that many activators of the innate immune system have not yet been paired with TLR family members; we expect progress with this soon. For example, recent work has implicated TLRs in pro-inflammatory responses induced by bacterial DNA (A. Ozinsky *et al.*, unpublished observations). More interesting perhaps are the observations that different PAMPs are recognized by distinct combinations of TLRs, suggesting that TLRs can establish a combinatorial repertoire to discriminate between the large number of PAMPs found in nature (A. Ozinsky *et al.*, unpublished observations). In fact the number of possibilities for pairing of TLRs is enormous. Moreover, one must also consider the possibility of the existence of other MD-2-like molecules that might regulate specific functions of TLRs.

The phagocytosis of bacteria and other pathogens by macrophages is one of the initiating events of the innate immune response. During phagocytosis, TLRs are recruited to the phagosomes, where they sample the contents and determine the nature of the pathogen[10]. Thus specific TLRs might distinguish between components in the phagosome and participate in the formulation of an inflammatory response appropriate for defence against a specific pathogen.

It is still unknown whether the TLRs directly bind PAMPs in mammalian cells. In *Drosophila* this seems to occur indirectly: microbial pathogens activate a protease cascade that generates a peptide ligand for the receptor[1]. The ligand of dToll has been defined as a proteolytic cleavage product named spaetzle[45]. It is conjectured that this polypeptide binds to dToll although there are at present no direct data to prove this. Moreover, the ligand for 18-wheeler has not yet been identified. Does such a model describe events at the cell surface of myeloid lineage cells that have been exposed to bacterial LPS? This seems unlikely but it must still be considered. Two recent reports suggest that LPS binds TLR4 directly[7,8]. As mentioned above, the active moiety of LPS is a glycolipid known as lipid A. Lipid IVa is a partial structure of lipid A with the peculiar property that it acts as an agonist of pro-inflammatory responses in the mouse and as an antagonist in humans. Transfection of mouse (or hamster) TLR4 into human macrophages gives them the ability to detect lipid IVa as an agonist, whereas transfection of mouse macrophages with human TLR4 has the opposite result[7,8]. Thus TLR4 alone determines how the macrophage responds to lipid IVa. This is hard to reconcile with the *Drosophila* model with an upstream proteolytic cascade. Perhaps the proteolytic cascade found in *Drosophila* is reflected by the complement and coagulation cascades of mammalian species. These latter systems are known to be activated directly by pathogens releasing peptidic products that act via receptor systems distinct from the TLRs.

TLR signalling pathways

Signalling via the TLRs has been studied by several groups, primarily in systems dependent on TLR2 and TLR4[26,46–49]. A framework that has guided studies of TLR signalling was provided by findings from investigations of signalling via the related IL-1 receptor (IL-1R) family[50,51]. The similarities between these two classes of receptor no doubt derive from the common Toll homology domain present in the cytoplasmic tails. Most studies have focused on the activation of TLRs leading to NF-κB translocation and transactivation, although other events such as the activation of the mitogen-activated protein (MAP) 3-kinase pathway are also triggered after TLR ligation. Both genetic and biochemical data support the scheme shown in Fig. 2 and discussed below.

Ligation of a TLR promotes dimerization and results in the recruitment of MyD88, which contains two domains: a C-terminal Toll homology domain that interacts with the Toll homology domain of the receptor, and an N-terminal death domain[26,48]. This death domain undergoes homophilic interaction with the death domain of a serine/threonine protein kinase known as IRAK; this leads to the autophosphorylation of IRAK[26,48]. Autophosphorylated IRAK then forms a complex with TRAF6 and this, in turn, results in the oligomerization of TRAF6. At this point the details of the pathway become less clear. Somehow the oligomerization of TRAF6 activates TAK-1, a member of the MAP 3-kinase family[52], and this leads to the activation of the IκB kinases. These kinases, in turn, phosphorylate IκB, leading to its proteolytic degradation and the translocation of NF-κB to the nucleus[28,29,46–48]. Concomitantly, members of the activator protein-1 (AP-1) transcription factor family, Jun and Fos[52–54], are activated, and both AP-1 transcription factors and NF-κB are required for cytokine production. Figure 2 also includes other components that have been identified by biochemical and genetic approaches but whose precise location within the pathway has yet to be defined. Although gene deletion studies have supported this activation pathway[34,55–57], there is clearly substantial additional complexity. For example, many components of the signalling pathway have homologues: at least three IRAK homologues have been demonstrated, and these are known to compensate partly for each other[58]. Interestingly, although no homologue of MyD88 has yet been found, deletion of this gene does not completely abolish the response to LPS, although the response is substantially delayed[55]. The explanation for the residual responsiveness in currently not known.

Logically, one might assume that downstream events also differ depending on which of the Toll receptors is ligated. The best-studied examples come from *Drosophila*, in which signalling through dToll and 18-wheeler have been shown to produce a distinct pattern of gene products[19,20]. Much less is known about mammalian systems other than that differential downstream events are linked to signalling through different TLRs. This will be a fruitful area of study in the future, for refined genomic approaches.

The past decade has thus been filled with remarkable advances in our understanding of the mechanisms of innate immune responses to microbial pathogens. In large part the progress was driven by a desire to understand the pathogenesis of a serious clinical syndrome known as septic shock. These advances encompass the identification of membrane receptors for specific products of microbial pathogens, the identification of essential components of intracellular signalling pathways and the characterization of the key mediators produced by cells of the innate immune system in response to infection. This body of information suggests that we will be able further to determine the genetic basis of disease susceptibility and outcome through studies of the genetic variation of essential components. For example, future studies will undoubtedly include a careful analysis of polymorphisms of TLRs. The latter might permit a correlation to be made between polymorphisms and disease outcome in patients in hospital at risk of septic shock. Other important advances will come from a better understanding of how different TLRs interact to recognize microbial pathogens and from a determination of the spatial organization of the various proteins comprising a receptor for a specific class of products released by pathogens. □

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Activation of Macrophages by Linear (1→3)-β-D-Glucans

IMPLICATIONS FOR THE RECOGNITION OF FUNGI BY INNATE IMMUNITY*

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Although (1→3)-β-D-glucans, which are one of major fungal cell wall components, are known to activate invertebrate innate immune systems, their activities on mammalian cells remain elusive. Here, we report their activities on mouse macrophages. Among the various (1→3)-β-D-glucans, curdlan, a linear (1→3)-β-D-glucan, although not branched β-glucans, exhibits significant activity to stimulate nuclear factor-κB in macrophages. The activity of curdlan is dramatically enhanced by pretreatment with sodium hydroxide or dimethyl sulfoxide, which disrupts multiple-stranded helices of (1→3)-β-D-glucans, and is dose-dependently inhibited by a (1→3)-β-D-glucan-binding protein and by laminarioligosaccharides with (1→3)-β-D-glucosidic linkages. Intriguingly, the activity of curdlan is also augmented by incubation with zymolyase, which releases (1→3)-β-D-glucans with a single helical structure from the glucan-networks assembled by multiple-stranded helices. The activation of macrophages culminates in the production of inducible nitric-oxide synthase, tumor necrosis factor-α, and macrophage inflammatory protein-2. Furthermore, a dominant-negative mutant of MyD88, an adaptor protein mediating signaling through the Toll-like receptor/interleukin-1 receptor-like (TIR) domain, inhibits the activation of macrophages by curdlan. These results strongly suggest that macrophages respond to linear (1→3)-β-D-glucans, possibly released from fungal cell walls, via a receptor(s) harboring the TIR domain, such as a Toll-like receptor, to induce inflammatory reactions.

Innate immune systems respond to characteristic molecules on microorganisms. Such molecules are indispensable structural components for the survival of microorganisms, and their presence in large numbers makes them ideal targets for recognition by innate immune systems, which utilize limited numbers of germ line-encoded proteins. The target molecules, represented by lipopolysaccharide (LPS)[1] and peptidoglycan (PGN) on Gram-negative and -positive bacteria, are called pathogen-associated molecular patterns (PAMPs) (1, 2). Recent studies have revealed that diverse molecules on bacteria or virus, including lipoprotein/peptides, flagellin, CpG DNA, and double-stranded RNA, function as PAMPs to stimulate the mammalian innate immune system via specific Toll-like receptors (TLRs), mammalian homologues of the *Drosophila* membrane protein Toll (3–9). Despite the intensive investigation of the responses to bacteria, much less is known about the responses to fungi, another important pathogen for multicellular organisms. The phagocytic responses and subsequent inflammatory reactions of macrophages to zymosan, a yeast cell wall component, indicate that the mammalian innate immune system has the capacity to respond to fungi (10). However, identification of PAMPs on fungi remains elusive because zymosan is a crude mixture of glucans, mannan, proteins, chitin, and glycolipids (11).

Among the fungal cell wall components, glucans with (1→3)-β-glucosidic linkages, (1→3)-β-D-glucans, are most abundantly present and provide mechanical strength to the cell walls (12). In addition to fungi, (1→3)-β-D-glucans are widely distributed in algae and higher plants, but rarely found in animals (12). In invertebrates, (1→3)-β-D-glucans are known to be potent stimulators for the innate immune system. In horseshoe crabs, factor G, a (1→3)-β-D-glucan-sensitive serine protease, is activated by the glucan to induce hemolymph coagulation (13–17). (1→3)-β-D-Glucans also activate the prophenoloxidase-activating cascade that leads to melanin formation in insects and crayfish (18). In plants, (1→3)-β-D-glucans are one of the elicitors to induce phytoalexin production (19, 20).

In mammals, (1→3)-β-D-glucans are known to be potent activators of the complement system (21). Furthermore, previous reports indicate that (1→3)-β-D-glucans exert inhibitory activities against tumor growth (22–25), in addition to exhibiting anti-inflammatory activities (26, 27). Because these activities are considered to be expressed through the stimulation of the reticulo-endothelial system, including stimulation of macrophages, endothelial, and reticulum cells, (1→3)-β-D-glucans are known as biological response modifiers (BRMs). In contrast to these activities observed *in vivo*, however, relatively little is known about the activities of (1→3)-β-D-glucans *in vitro*, and hence the molecular mechanisms of the cellular activation by (1→3)-β-D-glucans are poorly understood. Although some reports have described that (1→3)-β-D-glucans elicit the produc-

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[1] The abbreviations used are: LPS, lipopolysaccharide; PGN, peptidoglycan; PAMP, pathogen-associated molecular pattern; TLR, Toll-like receptor; BRM, biological response modifier; NF-κB, nuclear factor-κB; TIR, Toll/interleukin-1 receptor-like; GST, glutathione *S*-transferase; DMEM, Dulbecco's modified Eagle's medium; FCS, fetal calf serum; iNOS, inducible nitric-oxide synthase; TNF, tumor necrosis factor; MIP-2, macrophage inflammatory protein-2; Me_2SO, dimethyl sulfoxide; GBD, glucan-binding domain; DP, degree of polymerization.

tion of cytokines and nitric oxide (28–30), the relationships between the structures of the glucans and their stimulatory activities are still controversial due to a lack of reproducible *in vitro* systems to evaluate the activities of (1→3)-β-D-glucans.

In the present study, we evaluate the activities of (1→3)-β-D-glucans with a nuclear factor-κB (NF-κB) reporter system constructed with macrophages. This reliable and reproducible *in vitro* system allows characterization of the responses of macrophages to (1→3)-β-D-glucans. The results obtained indicate that the linear (1→3)-β-D-glucan curdlan exhibits significant cell-stimulating activities, and that the activities of (1→3)-β-D-glucans are dependent on their lengths and conformations. Furthermore, the analysis of the intracellular signaling suggests the involvement of MyD88, and therefore of a Toll/interleukin-1 receptor-like (TIR) domain-containing receptor(s), in the responses to (1→3)-β-D-glucans.

EXPERIMENTAL PROCEDURES

Reagents—Curdlan, LPS from *Escherichia coli* 0111:B4, PGN from *Staphylococcus aureus*, and laminarin from *Laminaria digitata* were purchased from Wako Pure Chemical Industries, Ltd. (Osaka, Japan), List Biological Laboratories, Inc. (Campbell, CA), Fluka Chemika-Biochemika (Buchs, Switzerland), and Sigma Chemical Co., respectively. Polymyxin B sulfate, poly(I)·poly(C), and mouse interferon-γ were from Sigma, Amersham Biosciences, and Genzyme Corp. (Cambridge, MA), respectively, and laminarioligosaccharides and zymolyase were from Seikagaku Corp. (Tokyo, Japan). LPS concentration in curdlan was measured by means of an LPS-specific Limulus amebocyte lysate (LAL) test using an Endospecy kit (Seikagaku Corp.). Glutathione *S*-transferase (GST) and a GST fusion protein with the xylanase Z-like domain of the horseshoe crab factor G were prepared as described previously (15). An expression plasmid for a dominant-negative mutant for MyD88 (pcDNA3-hMyD88C) was constructed by inserting a cDNA fragment for human MyD88 (amino acid 155 to the COOH terminus) created by a polymerase chain reaction into pcDNA3 (Invitrogen Life Technologies, Carlsbad, CA). pELAM1-Luc (31) and pRL-TK (Promega Corp., Madison, WI) were used as an NF-κB reporter plasmid and an internal control plasmid, respectively.

Cells—RAW264.7 and RAW-R12 cells were cultured in Dulbecco's modified Eagle's medium (DMEM) containing 10% heat-inactivated fetal calf serum (FCS) supplemented with 100 units/ml penicillin and 100 μg/ml streptomycin. RAW-R12 cells were obtained by stably transfecting pELAM-1-Luc into RAW264.7 cells.

NF-κB Reporter Assay—RAW-R12 cells (1×10^5 cells/well), stably transfected with the NF-κB reporter plasmid pELAM1-Luc, were seeded in a 96-well plate on the day before stimulation. The cells were stimulated as indicated and lysed, and their luciferase activities were measured by using a luciferase assay system (Promega Corp.). RAW264.7 cells (5×10^5 cells/well) in a 24-well plate were transfected with an expression plasmid together with an NF-κB reporter, pELAM1-Luc, and a control *Renilla* luciferase reporter, pRL-TK, using FuGENE 6™ transfection reagent according to the manufacturer's instructions (Roche Diagnostics). The cells were stimulated as indicated and lysed, and their luciferase activities were measured by using a dual-luciferase reporter assay system (Promega Corp.). The NF-κB reporter activity was divided by the activity of the *Renilla* control reporter to normalize transfection efficiency.

Western Blotting—Cells were stimulated as indicated and lysed in a buffer containing 50 mM Tris-HCl (pH 7.5), 0.15 M NaCl, 1% Nonidet P-40, 2 mM EDTA, 50 mM NaF, and 0.2 mM Na_3VO_4, supplemented with Complete™ protease inhibitor mixture (Roche Diagnostics) on ice for 30 min. After centrifugation, the cell lysate was immunoblotted with anti-inducible nitric-oxide synthase (iNOS) monoclonal antibody (Santa Cruz Biotechnology, Inc., Santa Cruz, CA).

Tumor Necrosis Factor (TNF)-α Bioassay—TNF-α secreted from stimulated cells into culture medium was assayed by bioassay using L929 cells (32). The standard curve was obtained with recombinant mouse TNF-α (Genzyme Techne). It was confirmed that more than 95% of TNF-α activities in the sample were neutralized with anti-mouse TNF-α monoclonal antibody (Genzyme Techne).

Northern Blot Analysis—After RAW264.7 cells were stimulated, total RNA was extracted from the cells by using TRIzol™ (Invitrogen Life Technologies). 10 μg of RNA was subjected to electrophoresis on a 1.2% agarose gel, transferred to Hybond™-N+ nylon membrane (Amersham Biosciences), and hybridized with a ^{32}P-labeled probe for iNOS, TNF-α, or macrophage inflammatory protein-2 (MIP-2). As a loading control, the same blot was hybridized with a probe for glyceraldehyde-3-phosphate dehydrogenase.

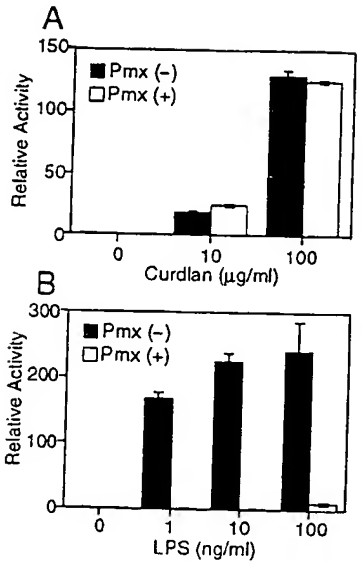

FIG. 1. **Curdlan stimulates NF-κB activity of macrophages.** RAW-R12 cells (1×10^5 cells), stably transfected with an NF-κB reporter, were stimulated by curdlan (*A*) or LPS (*B*) at the indicated concentrations in the presence (+) or absence (−) of 100 units/ml polymyxin B (*Pmx*). Curdlan was dissolved in 0.1 M NaOH and diluted 100-fold with DMEM containing 10% FCS to give the indicated final concentration before addition to the cells. After incubation at 37 °C for 6 h, the cells were lysed and the luciferase activities were measured. Relative activities to that of unstimulated cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of two independent experiments.

RESULTS

Curdlan, a Linear (1→3)-β-D-Glucan Preparation, Activates Macrophages—To evaluate the activity of (1→3)-β-D-glucans on the mammalian cells, we assessed NF-κB activation in macrophages, which is one of the prominent responses of the mammalian innate immune system. Among various (1→3)-β-D-glucan preparations examined, curdlan, a linear (1→3)-β-D-glucan derived from *Alcaligenes faecalis* var. *myxogenes* 10C3K (33), at final concentrations of 10–100 μg/ml, induced robust activation of NF-κB in the mouse macrophage cell line RAW264.7, which was stably transfected with an NF-κB reporter plasmid (RAW-R12) (Fig. 1*A*). Because curdlan is insoluble at neutral pH, it was once solubilized in NaOH solution and then added to the culture medium to neutralize pH by diluting 100-fold. The NaOH-pretreated curdlan was added to the cells for stimulation immediately after the neutralization. When the contamination of the curdlan preparation with LPS, a strong activator of macrophages, was measured by the LPS-specific Limulus amebocyte lysate (LAL) test, it was revealed that <1 pg of LPS was present per microgram of curdlan (data not shown). To rule out the possibility that the NF-κB-stimulating activity was due to contaminating LPS, the cells were stimulated in the presence of polymyxin B, which neutralizes the LPS activity (34). Polymyxin B effectively inhibited the activity of 100 ng/ml LPS (Fig. 1*B*), but not the activity of curdlan (Fig. 1*A*). In all of the following experiments, stimulation by curdlan was done in the presence of polymyxin B.

We carefully titrated the concentrations of NaOH used for solubilizing curdlan (Fig. 2*A*). Curdlan was solubilized with NaOH at the indicated concentrations, then diluted 100-fold with the medium and used to stimulate the cells. NaOH alone at 0.01–0.3 M did not stimulate the NF-κB activity (data not

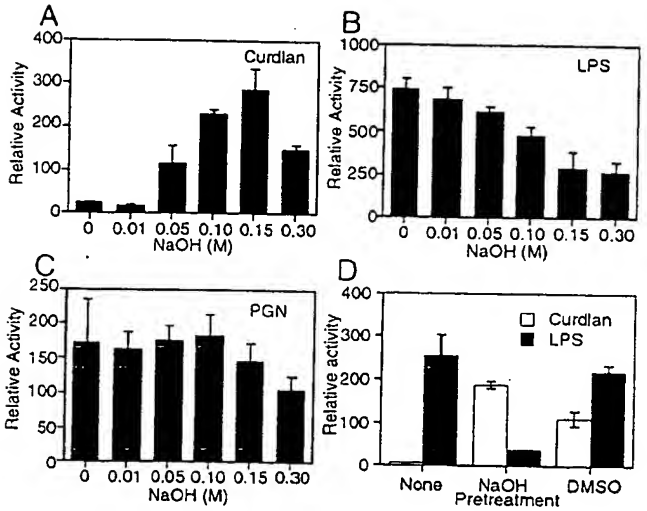

FIG. 2. **The activity of curdlan is enhanced by pretreatment with NaOH or Me_2SO.** Curdlan (*A*), LPS (*B*), or PGN (*C*) was dissolved in NaOH at the indicated concentrations. Then, the samples were diluted 100-fold with DMEM containing 10% FCS before stimulation of RAW-R12 cells (1×10^6 cells), stably transfected with an NF-κB reporter. In *D*, curdlan or LPS was dissolved in 0.15 M NaOH or Me_2SO and then diluted 100-fold with the medium. To simultaneously compare the effects of the pretreatment with NaOH and Me_2SO, the stimulants pretreated with NaOH or Me_2SO were diluted with the medium containing Me_2SO or NaOH, respectively, to keep the final concentrations of NaOH and Me_2SO constant. In the experiments without the pretreatment (*None*), the stimulants in water were diluted with the medium containing both NaOH and Me_2SO. The final concentrations of NaOH and Me_2SO in the medium were kept constant. The final concentrations of curdlan, LPS, and PGN were 100 μg/ml, 1 ng/ml, and 100 μg/ml, respectively. Stimulation by curdlan was carried out in the presence of 100 units/ml polymyxin B. After incubation at 37 °C for 6 h, the cells were lysed and the luciferase activities were measured. Relative activities to that of unstimulated cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of three independent experiments.

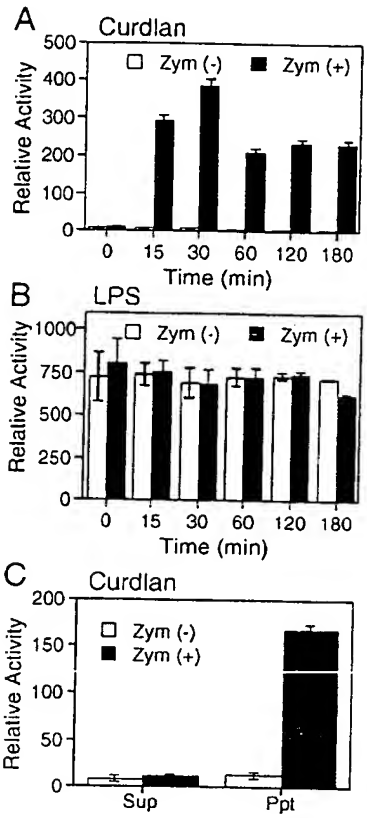

FIG. 3. **Zymolyase treatment of curdlan enhances its activity.** Curdlan (*A*) or LPS (*B*) was dissolved in phosphate-buffered saline with or without 0.5 mg/ml zymolyase (*Zym*) and incubated at 37 °C for the indicated time. Then the zymolyase-treated curdlan and LPS were diluted 100-fold with DMEM containing 10% FCS to give the final concentrations of 100 μg/ml and 1 ng/ml, respectively, and added to RAW-R12 cells (1×10^5 cells), stably transfected with an NF-κB reporter. *C*, curdlan treated with zymolyase for 30 min was centrifuged at 20,000 × *g* for 15 min to obtain the soluble fraction (*Sup*). The resulting precipitates (*Ppt*) were washed three times with phosphate-buffered saline and then suspended in the original volume of the same buffer. The obtained soluble and insoluble fractions were diluted 100-fold with the medium and used to stimulate the cells. Stimulation by curdlan was carried out in the presence of 100 units/ml polymyxin B. After incubation at 37 °C for 4 h, the cells were lysed, and the luciferase activities were measured. Relative activities to that of unstimulated cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of four independent experiments.

shown). Insoluble curdlan suspension in water (0 M NaOH) showed weak activity, and this activity was significantly augmented by increasing the concentrations of NaOH. The addition of 0.15 M NaOH to the medium raised the pH of the medium by 0.2–0.3 units, but the viability of the cells did not change during the assay (data not shown). Although the activity was reduced with 0.3 M NaOH, this reduction was most likely caused by the toxic effects of the increased pH of the medium on the cells: when NaOH-pretreated curdlan was diluted 1,000-fold with the medium, the NF-κB-stimulating activity of curdlan pretreated with 0.3 M NaOH was higher than that of curdlan pretreated with 0.15 M NaOH (data not shown). When LPS was similarly treated with NaOH, its ability to activate the NF-κB of macrophages was gradually reduced by increasing concentrations of NaOH (Fig. 2*B*). The NF-κB-stimulating activity of PGN, which is insoluble even in the NaOH solutions, was scarcely affected by NaOH, but was also inhibited at higher NaOH concentrations (Fig. 2*C*). When KOH was used instead of NaOH, similar augmentation of the curdlan activity and inhibition of the LPS and PGN activities were observed (data not shown). We also found that another preparation of a linear (1→3)-β-D-glucan, paramylon, activated the cells to a lesser degree, and in an NaOH-dependent manner like that of curdlan (data not shown). Curdlan solubilized in dimethyl sulfoxide (Me_2SO) also exhibited the enhanced activity as in NaOH (Fig. 2*D*).

We next tried to activate the insoluble curdlan without the pretreatment with NaOH or Me_2SO, since these compounds do not occur under physiological conditions. We tested the effect of treatment with zymolyase, an endoglucosidase, which partially solubilizes curdlan. Zymolyase treatment for 15–30 min markedly stimulated the activity of curdlan (Fig. 3*A*). Longer treatment with zymolyase failed to completely solubilize curdlan, the activity of which was slightly reduced at 60 min and sustained up to 24 h. The effect of zymolyase treatment was specific to curdlan, because zymolyase itself did not activate NF-κB in the absence of curdlan (data not shown), and the enzyme treatment did not affect the activity of LPS (Fig. 3*B*). When the zymolyase-treated curdlan was centrifuged, the activity was found in the insoluble fraction, and the soluble fraction showed little activity (Fig. 3*C*). Zymolyase treatment of paramylon for 15 min also enhanced its NF-κB-stimulating activity, which was gradually decreased by longer treatment with zymolyase (data not shown).

Because the NaOH and zymolyase treatments convert curdlan from insoluble particulates to soluble glucans, it is possible that these treatments release unknown contaminants in the particulates that activate macrophages. We attempted to determine if the macrophage-stimulating activity is due to (1→3)-

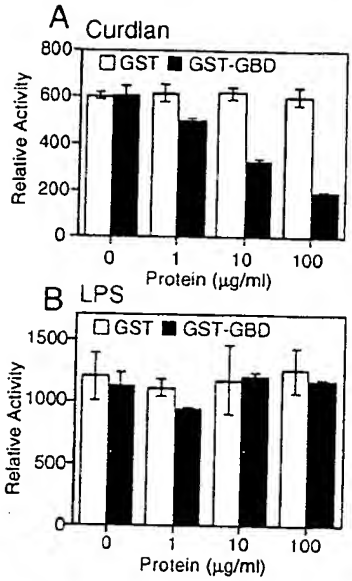

FIG. 4. **A β-glucan-binding protein inhibits the activity of curdlan.** Curdlan pretreated with 0.15 M NaOH (*A*) or LPS (*B*) was diluted 100-fold with DMEM containing 10% FCS and the indicated concentrations of *GST* or a GST fusion protein of GBD of horseshoe crab factor G (*GST-GBD*), and was incubated at 37 °C for 30 min. The final concentrations of curdlan and LPS were 100 μg/ml and 1 ng/ml, respectively. The preincubated curdlan or LPS was added to stimulate RAW-R12 cells (1×10^5 cells), stably transfected with an NF-κB reporter, at 37 °C for 6 h. Then, the cells were lysed, and the luciferase activities were measured. Stimulation by curdlan was carried out in the presence of 100 units/ml polymyxin B. Relative activities to that of unstimulated cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of three independent experiments.

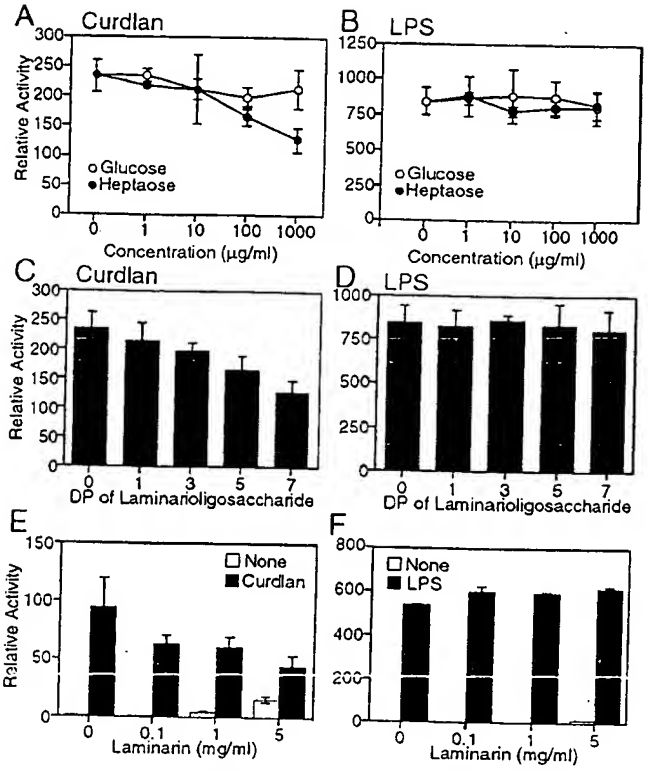

FIG. 5. **Short (1→3)-β-D-glucans inhibit the activity of curdlan.** RAW-R12 cells (1×10^5 cells), stably transfected with an NF-κB reporter, were preincubated with DMEM containing 10% FCS and *glucose* or laminarioheptaose (*Heptaose*) (*A* and *B*), laminarioligosachharides with varying DP (1 mg/ml) (*C* and *D*), or laminarin (*E* and *F*) at 37 °C for 30 min. Then, curdlan pretreated with 0.15 M NaOH (*A*, *C*, and *E*) or LPS (*B*, *D*, and *F*) was directly added to the medium to give the final concentration of 100 μg/ml or 1 ng/ml, respectively. In the experiments in *E* and *F*, 0.15 M NaOH alone was used as a negative control (*None*). After incubation at 37 °C for 6 h, the cells were lysed and the luciferase activities were measured. Stimulation by curdlan was carried out in the presence of 100 units/ml polymyxin B. Relative activities to that of unstimulated cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of four independent experiments.

β-D-glucan itself by using a (1→3)-β-D-glucan-binding protein. The COOH-terminal xylanase Z-like domain of the (1→3)-β-D-glucan-sensitive horseshoe crab factor G binds specifically to a (1→3)-β-D-glucan disaccharide at a K_a of 5.77×10^7 M^{-1} and neutralizes the activity of (1→3)-β-D-glucan to activate factor G (15). We utilized this glucan-binding domain (GBD) of the horseshoe crab factor G to examine the specificity of the activity of curdlan. When preincubated with a glutathione *S*-transferase (GST) fusion protein containing GBD (*GST-GBD*), the activity of NaOH-pretreated curdlan was dose-dependently inhibited (Fig. 4*A*). The inhibition was not observed with GST. In contrast to the effect on curdlan, the activity of LPS was not affected either by GST-GBD or GST (Fig. 4*B*). Neither GST nor GST-GBD activated NF-κB in the absence of curdlan (data not shown). Thus, it was confirmed that the activity of curdlan was indeed due to the authentic (1→3)-β-D-glucan.

Curdlan-mediated Activation Is Inhibited by Shorter (1→3)-β-D-Glucans—Because curdlan is a long (1→3)-β-D-glucan with a degree of polymerization (DP) of ~500, we next investigated the activities of short (1→3)-β-D-glucans. When the activities of laminarioligosaccharides, linear water-soluble (1→3)-β-D-glucans with a DP of 2–7, were examined by using RAW-R12 cells, none of them exhibited the stimulating activity at concentrations up to 1 mg/ml even after the NaOH pretreatment (data not shown). Instead, laminarioheptaose (DP = 7) inhibited the curdlan-mediated activation in a dose-dependent manner (Fig. 5*A*). Laminarioheptaose did not inhibit LPS-mediated activation, however (Figs. 5*B* and *D*). When laminarioligosaccharides with different lengths were examined, the inhibition was dependent on the DP of the oligosaccharides, with the longer oligosaccharides exhibiting the greater inhibition (Fig. 5*C*). We further examined the activity of laminarin derived from *L. digitata*, which is a water-soluble (1→3)-β-D-glucan preparation with a DP of 20–30, and with branches of single glucosyl residues by a (1→6)-β-linkage (35). Laminarin exhibited a weak stimulating activity at 1–5 mg/ml. Nevertheless, it dose-dependently inhibited the curdlan-mediated activation without affecting the LPS-mediated activation (Fig. 5, *E* and *F*).

Curdlan Induces Production of Proinflammatory Mediators—We next investigated whether the stimulation of macrophages with curdlan leads to inflammatory reactions. We treated thioglycolate-elicited peritoneal macrophages with curdlan or LPS and examined the induction of iNOS by Western blotting. 16 h after stimulation with curdlan, a significant induction of iNOS was observed (Fig. 6*A*). Like the induction by LPS, the induction by curdlan was considerably enhanced by co-stimulation with interferon-γ. Polymyxin B inhibited the induction by LPS but not that by curdlan. The induction of iNOS was also observed in bone marrow-derived macrophages (data not shown) and RAW264.7 cells (Fig. 6*B*) by the treatment with curdlan alone. When the time course of the iNOS induction was examined, the induction by curdlan was much slower than that by LPS (Fig. 6*B*). We also measured the TNF-α production in the cells stimulated by curdlan or LPS. In contrast to the induction of iNOS, curdlan alone induced TNF-α secretion comparable to that by LPS with a similar time course (Fig. 6*C*).

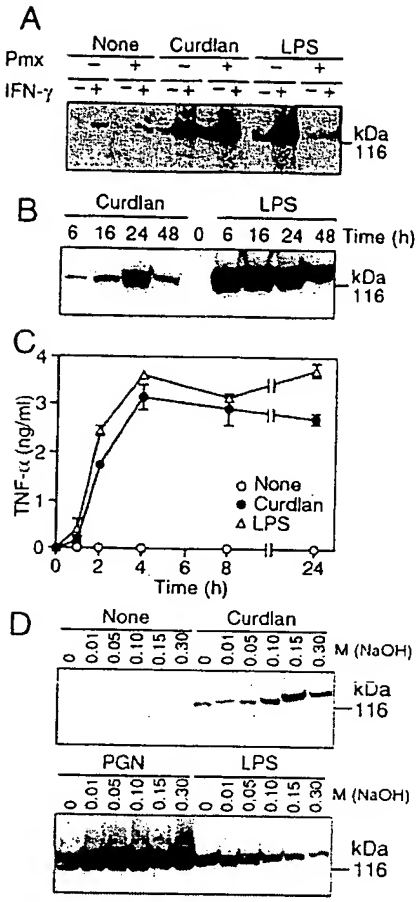

FIG. 6. **Curdlan induces iNOS and TNF-α.** *A*, thioglycolate-elicited peritoneal macrophages were stimulated with 100 μg/ml curdlan pretreated with 0.15 M NaOH or 1 ng/ml LPS in the presence (+) and absence (−) of 10 units/ml interferon-γ (*IFN-γ*) and 100 units/ml polymyxin B (*Pmx*) at 37 °C for 16 h. *B*, RAW264.7 cells were stimulated with 100 μg/ml curdlan pretreated with 0.15 M NaOH or 1 ng/ml LPS at 37 °C for the indicated time. Then the cell lysate was prepared and analyzed by Western blotting with an anti-iNOS antibody. *C*, RAW264.7 cells were stimulated with 100 μg/ml curdlan pretreated with 0.15 M NaOH or 100 ng/ml LPS for the indicated time. TNF-α in the culture supernatant was measured by an L929 cytotoxicity assay. Data are shown as the mean ± S.E. of duplicate samples. *D*, curdlan, LPS, and PGN were pretreated with the indicated concentrations of NaOH. They were then diluted 100-fold with DMEM containing 10% FCS and added to RAW264.7 cells for stimulation at 37 °C for 16 h. The final concentrations of curdlan, LPS, and PGN were 100 μg/ml, 1 ng/ml, and 100 μg/ml, respectively. The cell lysate was analyzed by Western blotting with an anti-iNOS antibody. Data shown are representative of at least three independent experiments.

When the effect of the NaOH pretreatment on the iNOS induction was examined, the induction was found to be dependent on the concentrations of NaOH: the maximum induction was observed when curdlan was pretreated with 0.15 M NaOH (Fig. 6*D*). The activity of LPS was reduced by higher concentrations of NaOH and that of PGN was less affected. These results coincide well with the effect of NaOH on the NF-κB activation.

To determine whether the inductions by curdlan occur at the mRNA level, we analyzed changes of mRNAs for iNOS and TNF-α after the stimulation (Fig. 7). The induction of iNOS mRNA by curdlan was slower and much weaker compared with that by LPS. On the other hand, the curdlan-stimulated cells exhibited a rapid and strong induction of TNF-α mRNA, which was comparable to that of the LPS-stimulated cells. These results are consistent with the pattern of the protein expression shown in Fig. 6. We also found that mRNA for macrophage inflammatory protein (MIP)-2, another inflammatory mediator, was also induced by curdlan as strongly as by LPS. In contrast to mRNA for iNOS or TNF-α, the MIP-2 mRNA decayed faster in curdlan-stimulated cells than in LPS-stimulated cells.

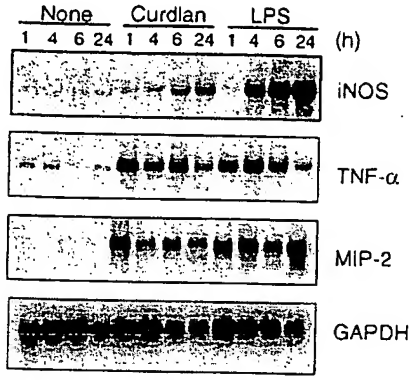

FIG. 7. **Curdlan induces mRNAs for iNOS, TNF-α, and MIP-2.** RAW264.7 cells were stimulated with 100 μg/ml curdlan pretreated with 0.15 M NaOH or 1 ng/ml LPS for the indicated time. Total RNA was extracted from the cells and subjected to Northern blotting analysis with a probe for *iNOS*, *TNF-α*, *MIP-2*, or glyceraldehydes-3-phosphate dehydrogenase (*GAPDH*). Data shown are representative of two independent experiments.

Cellular Activation by Curdlan Is Mediated through a MyD88-dependent Pathway—Curdlan stimulation of macrophages culminates in the production of inflammatory mediators that are also produced by LPS- or PGN-stimulation, whose signaling is transduced into the cells through TLR4 or TLR2, respectively (4, 36, 37). Although TLR2- or TLR4-transfected HEK293 cells did not respond to curdlan,[2] we suspected that curdlan might activate other TLRs. All the TLRs thus far identified utilize the adaptor protein MyD88. The truncated mutant of MyD88 harboring the COOH-terminal region interacts with the cytoplasmic domain of TLRs and hence acts as a dominant-negative mutant (38, 39). We used this mutant to determine whether curdlan-mediated signaling is also mediated by MyD88. When the activation of NF-κB was evaluated on vector- or the MyD88 mutant-transfected RAW264.7 cells, the curdlan-mediated activation was significantly inhibited in the mutant-transfected cells, as were the LPS- and PGN-mediated activations (Fig. 8). On the other hand, transfection of the MyD88 mutant was ineffective in inhibiting the activation by the double-stranded RNA poly(I)·poly(C), as shown in previous reports (40, 41). The results indicate that curdlan-mediated signaling is also mediated through the MyD88-dependent pathway.

DISCUSSION

After screening of various (1→3)-β-D-glucans by measuring NF-κB-activating activity in macrophages, we identified curdlan, a linear (1→3)-β-D-glucan, as the strongest activator. The glucans screened by the current system included branched (1→3)-β-D-glucans such as laminarin, paramylon, schizophyllan, lentinan, bakers' yeast β-glucan, barley β-glucan, and krestin. Although schizophyllan, lentinan, and krestin are known as BRM and have been reported to induce the production of nitric oxide and/or TNF-α in macrophages (28, 42, 43), by our present assay system we could not detect significant NF-κB-stimulating activities of these branched (1→3)-β-D-glucans at concentrations of 100 μg/ml with or without NaOH pretreatment. Instead, another linear (1→3)-β-D-glucan,

[2] K. Kataoka and T. Muta, unpublished data.

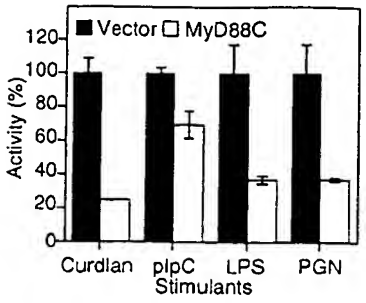

FIG. 8. **Curdlan-mediated signaling is inhibited by a dominant-negative mutant of MyD88.** RAW264.7 cells were transfected with an expression plasmid for a dominant-negative mutant of MyD88 (*MyD88C*) or an empty vector (*Vector*), together with an NF-κB reporter plasmid, pELAM1-Luc, and a control *Renilla* reporter plasmid, pRL-TK. The cells were stimulated with 100 μg/ml *curdlan* pretreated with 0.15 M NaOH, 10 μg/ml poly(I)·poly(C) (*pIpC*), 1 ng/ml *LPS*, or 100 μg/ml *PGN* at 37 °C for 6 h. Then the cells were lysed, and the luciferase activities were measured. The transfection efficiency was normalized by *Renilla* luciferase activity derived from co-transfected control vector, pRL-TK. Percent activities to that of vector-transfected cells are shown. Data are shown as the mean ± S.E. of duplicate samples and are representative of three independent experiments.

paramylon, activated NF-κB after the pretreatment with NaOH, but to a much lesser extent than curdlan.

Various microbe-derived molecules have been reported to activate innate immunity via respective specific TLRs (2). We carefully examined the possibility that this activity was due to a trace amount of contaminants derived from microbes in the curdlan preparation. Based on the following observations, we concluded that the activity of curdlan shown in the present study was due to the authentic (1→3)-β-D-glucan. The activity of curdlan was not affected by polymyxin B, which neutralized 100 ng/ml LPS effectively (Fig. 1). The activity of curdlan was dependent on the pretreatment with NaOH, whereas those of LPS and PGN were not affected or reduced by the pretreatment (Fig. 2). Furthermore, TLR2-, 3-, 4-, 5-, or 9-transfected HEK293 cells, which are responsive to PGN/lipopeptides, double-stranded RNA, LPS, flagellin, or CpG DNA, respectively (7–9, 31, 44, 45), did not respond to curdlan.[2] More convincing evidence was obtained when we tested the effect of the glucan-binding protein. GBD of the horseshoe crab factor G, which specifically binds to (1→3)-β-D-glucosidic linkages, inhibited the activity of curdlan but not that of LPS (Fig. 4). The modulatory effects of short (1→3)-β-D-glucans and zymolyase, an endo-(1→3)-β-D-glucosidase, on the activity of curdlan further supported the conclusion. Since this was the first careful examination of such contaminants, and since our results showed that some preparations of β-glucans contained polymyxin B-inhibitable activities (data not shown), a portion of the activities of (1→3)-β-D-glucans or their derivatives described in previous reports might be attributable to the contaminant(s) that stimulates macrophages.

Zymosan, which consists of yeast cell wall particles, is one of the strong macrophage activators containing (1→3)-β-D-glucans. In contrast to curdlan, zymosan activated TLR2-transfected HEK293 cells, and the activation of macrophages by zymosan was only weakly inhibited by the short (1→3)-β-D-glucans or GBD (data not shown). Thus, zymosan should contain multiple stimulators for macrophages in addition to (1→3)-β-D-glucans, and hence it is not an ideal material to study the activities of (1→3)-β-D-glucans.

The activity of curdlan was dramatically enhanced by the pretreatment with NaOH (Figs. 2 and 6*D*). Long gel-forming (1→3)-β-D-glucans are basically composed of a single helix in water, and the single helices of these molecules associate to form local junction zones composed of double- or triple-stranded helices, which are connected by the single helices to form networks (46). The addition of NaOH up to 0.19 M gradually breaks the multiple-helical structures without changing the single helical conformation. At NaOH concentrations between 0.19 and 0.22 M, a transition occurs from the single helix to random structures (47, 48). Pretreatment with Me_2SO, which converts the conformation of curdlan to random structures (49), also activated curdlan (Fig. 2*D*). The augmentation of the activity of curdlan by pretreatment with NaOH or Me_2SO indicates that the single helix and random structures are active conformations for the stimulation of NF-κB in macrophages. It has been reported that the horseshoe crab factor G is also activated by (1→3)-β-D-glucans with the single helical and random structures, but not by those with triple-stranded helices (50).

The zymolyase treatment, which is unlikely to change the conformation of curdlan, would release the single helices from the network of curdlan, since the enzyme is an endoglucanase that cleaves the single helices but not the multiple helices (51). The observation that the zymolyase treatment stimulated the activity of curdlan indicates that the molecular flexibility of the single helix, which is fixed by the multiple-stranded helices, is important for the activity of curdlan. Solubility seems not to be important for the activity of (1→3)-β-D-glucans, because even after the zymolyase treatment, the active fraction was in the insoluble fraction but not in the soluble fraction (Fig. 3*D*). Carboxymethylated curdlan is soluble in water, but inactive in our assay system (data not shown).

The activity of curdlan to stimulate NF-κB was inhibited by transfection of a MyD88 dominant-negative mutant containing the COOH-terminal TIR domain (Fig. 8). MyD88 acts as an essential adaptor protein proximal to interleukin-1 receptor and TLRs by associating the receptors through their TIR domains. Therefore, the inhibition by the MyD88 mutant strongly suggests that the signal transduction induced by (1→3)-β-D-glucans is mediated through a member(s) of the TLR family. Although several membrane components, such as complement receptor 3 (52), a scavenger receptor (53), lactosylceramide (54), and dectin-1 (55, 56), have been reported to bind to (1→3)-β-D-glucans, none of these proteins are likely to be inhibited by the MyD88 mutant. They might function as phagocytic receptors, although their significance in the activation of macrophages is unknown. The identification of the responsible TIR-containing receptor(s) for curdlan remains to be determined. The qualitatively different induction patterns of iNOS, TNF-α, and MIP-2 by curdlan (Figs. 6 and 7) suggest that the receptor for curdlan is distinct from that for LPS or PGN.

As in the cases of most PAMPs that stimulate TLRs, it is currently unknown whether or not curdlan directly binds to a cell surface receptor. Our characterization of curdlan-mediated activation of macrophages, however, provided some insight into the nature of the curdlan-recognizing protein. Water-soluble short linear (1→3)-β-D-glucan oligosaccharides with a DP of <7 did not activate macrophages at all; in fact, they inhibited the curdlan-mediated activation (Fig. 5). Thus, it is strongly suggested that the short (1→3)-β-D-glucans with the random structures bind to the curdlan-recognizing protein or receptor, but are too short to support the activation of this protein. Since linear (1→3)-β-D-glucans with a DP of <20 are soluble, and the soluble fraction of the zymolyase-treated curdlan did not exhibit the NF-κB- activating activity; the active glucans should have DPs of more than 20. Branched (1→3)-β-D-glucans as long as curdlan were incapable of inducing the NF-κB activation. It has been shown that activation of the horseshoe crab factor G is induced by the intermolecular interaction of factor G mole-

cules on a template of linear and sufficiently long (1→3)-β-D-glucans that support the collision of the molecules (14, 15). The curdlan-recognizing protein or receptor might be activated by a mechanism similar to that of factor G, thereby leading to the activation of macrophages.

Although many reports have described the activity of β-glucans as BRM *in vivo*, much less is known about the *in vitro* activity of (1→3)-β-D-glucans. This fact suggests that a complex system is required to express the activity of (1→3)-β-D-glucans. As we showed in this paper, the macrophage-stimulating activity of curdlan requires disassembly or disruption of the multiple-stranded helices at junction zones of the linear (1→3)-β-D-glucans. From a physiological or pathological point of views, it is an intriguing finding that curdlan was activated by zymolyase. On fungal cell surfaces, (1→3)-β-D-glucans exist as an insoluble matrix immobilized on the cell walls, which could be enzymatically released by a glucan-hydrolase(s) such as (1→3)-β-, (1→6)-β-, or (1→4)-β-D-glucosidase. The released fragments may be the activators for macrophages. In addition, some carrier proteins for the released insoluble fragments might be present. If the cells producing such glucosidases and/or carrier proteins are different from the cells activated by the modified (1→3)-β-D-glucans, it is not hard to imagine that a series of reactions would be difficult to reconstitute in the *in vitro* systems. Elucidation of the physiological or pathological modification of (1→3)-β-D-glucans, and the identification of the receptor responsible for the glucan-mediated signaling should lead to a better understanding of the responses of the innate immune system to fungi, as well as the development of a new therapeutic utilization of (1→3)-β-D-glucans as an effective BRM.

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REVIEW

Targeting epidermal Langerhans cells by epidermal powder immunization

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ABSTRACT

Immune reactions to foreign or self-antigens lead to protective immunity and, sometimes, immune disorders such as allergies and autoimmune diseases. Antigen presenting cells (APC) including epidermal Langerhans cells (LCs) play an important role in the course and outcome of the immune reactions. Epidermal powder immunization (EPI) is a technology that offers a tool to manipulate the LCs and the potential to harness the immune reactions towards prevention and treatment of infectious diseases and immune disorders.

Key words: *Langerhans cells, dendritic cells, vaccines, epidermal immunization.*

The biology of langerhans cells

LCs, like the related dendritic cells (DCs), are professional antigen presenting cells. LCs are uniquely present in the epidermis of the skin while DCs are present in the mucosa, dermis, and internal organs. Epidermal LCs form a semi-continuous network in the skin. The density of LCs in most areas of the human skin with the exception of sole and palm is approximately 500-1000 cells/mm^2 (an estimated total of 10^9 LCs for an adult)[1-3]. LCs represent a very dynamic cell population. At any given time, there are LCs loaded with antigens leaving the skin and fresh LCs coming in to replenish the sites. The mean residence time in skin for a LC is about 3 w[4, 5].

LCs initiate, maintain, and regulate adaptive immunities in the skin. These cells take up epicutaneous antigens, emigrate into the regional skin-draining lymph nodes, and present the processed antigens to the T cells. LCs are derived from bone marrow progenitor cells, which travel through blood to home in the epidermis and become LCs[6-8]. Cytokines such as interleukin-4 (IL-4), tumor-necrosis factorα (TNF-α), and granulocyte macrophage colony stimulating factor (GM-CSF) are thought to drive the differentiation process of the progenitor cells[9]. The resident LCs in the epidermis are immature cells possessing strong phagocytic and endocytic capacity. The primary function of the immature LCs is to capture and process antigens. After picking up antigen, LCs migrate across the basal membrane and dermis into draining lymph nodes via lymphatics. Migration is mediated by proinflammatory cytokines including TNF-α and IL-1 that originate from the LCs and other epidermal cells including keratinocytes[10, 11]. During migration, maturation takes place. Mature LCs express high levels of MHC class I and class II antigens, costimulatory molecules (CD80 and CD86), and chemokine receptors, all of which are important for its antigen presentation function[12-14]. Antigen presentation to T cells by LCs takes place in the draining lymph nodes. LCs are capable of presenting antigen to both the naive T cells and antigen-specific T cells

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of $CD4^+$ and $CD8^+$ phenotypes to stimulate both antibody and cellular immune responses. Induction of CTL responses in mice may require as few as 10 LCs, whereas the induction of an antibody response may require upto 1000 LCs[15, 16].

Cytokines secreted by LCs such as IL-1, IL-6, IL-12 and IL-18 play an important role in regulating the immune responses[11, 17, 18]. These cytokines combined with those secreted by other epidermal cells can polarize the LCs to selectively bias the development of Th1 or Th2 cells. For example, LCs promote the development of a Th1 type response in the presence of a high level of IL-12[19]. LCs matured in the presence of a high level of IL-10, or in the absence of IL-12, stimulate the maturation of Th2 type responses[20, 21]. Interaction of LCs with other cells of the immune system results in differential cellular and humoral immune responses and cytokine production (Fig 1) which are important in protection against infections, and sometimes, are associated with immune disorders including allergies[22].

Role of LCs/DCs in immunity and immune disorders

Infectious diseases

LCs play an important role in the immune responses to cutaneous infections caused by Herpes simplex virus types 1 and 2, poxvirus, papillomavirus, varicella virus, and other pathogens that cause skin infections[23]. LCs may contribute to the anti-infection immunity by at least two mechanisms, induction of cellular and humoral immunities by presenting antigen of the infectious agents to the immune system and secretion of cytokines that have direct antiviral activities[24]. Inoculation of HSV-1 or vaccinia viruses to a skin site depleted of LCs results in higher morbidity and mortality than a normal skin site in animal studies[25]. Vice versa, activation of LCs locally or systemically correlates with the resistance to infection with these viruses[26]. LCs not only contribute to the control of primary infections, but also play a role in the control of recurrent viral infections caused by HSV-1[23].

Allergic contact dermatitis (ACD)

ACD is mediated by responses of $CD4^+$ T cells to the contact allergens, mostly low-molecular-weight-haptens. ACD has two phases, sensitization and elicitation. Sensitization refers to events that lead to the activation of T cells whereas the elicitation refers the events that occur when the activated T cells are re-exposed to the same allergen. The sensitization and elicitation phases of ACD are complex processes that involve different cells and molecules[27-29]. However, LCs are clearly shown to play an important role in the sensitization process. When applied to the skin, contact allergens bind to the proteins in the epidermis and become a complete antigen. The complete antigen is picked up by LCs or dermal DCs. Some allergens may bind directly to the MHC class II molecules of LCs since topically administered contact allergens accumulate in the LCs of the local skin. Once the LCs have taken up a contact allergen, they migrate to the draining lymph node and present the allergen to the naive $CD4^+$ T cells. Activated $CD4^+$ T cells proliferate and home in the peripheral tissues including skin. In the elicitation phase (when exposed to the same allergen again), antigen presentation is still required, this may be accomplished by LCs and other skin cells. Some evidence suggests that allergen-specific T cells can be activated by allergen loaded LCs in the skin to release cytokines, which cause the infiltration of inflammatory cells.

Allergy and Asthma

Recent studies suggest that allergy and asthma are due to eosinophilic inflammation of the airway mediated by Th2 cytokines (IL-4, IL-5, IL-6, and IL-13 etc) (Fig 1) in response to environmental stimulants[30]. Airway DCs are believed to play an important role in the sensitization to asthma[31, 32]. The airway DCs reside in the lateral intercellular spaces of the basal epithelial cells and form a network of cells in a way similar to LCs in the skin. Inhaled allergens are captured, processed, transported to the regional lymph nodes and presented to naive T cells. The low levels or absence of IL-12 and the abundance of IL-4 and IL-10 cytokines are believed to be important in selectively biasing the development of Th2 cells. Some Th2 cytokines such as IL-3, IL-5 and GM-CSF cause airway eosinophilia, whereas other Th2 cytokines (IL-4, IL-13) cause the production of IgE antibodies by B cells. In the elicitation phase, allergen introduced cross-linking of antigen-specific IgE antibody bound to mast cells through

high-affinity receptors triggers mast cells to release inflammatory mediators and subsequent sustained infiltration of eosinophils. Increased LC density has been observed in the affected tissue during the allergic inflammation, which is thought to be important in controlling and maintaining the inflammation.

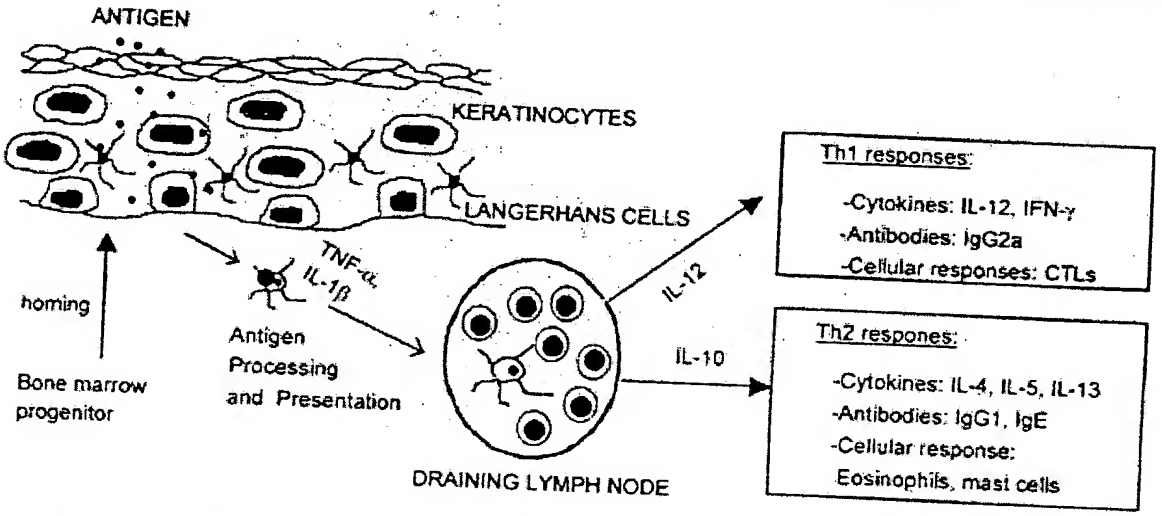

Fig 1. A schematic representation of the LCs' life circle and their function of presenting antigens introduced by the skin route. Bone marrow derived LC progenitors travel through blood and home in the epidermis. LCs take up antigens introduced via the skin route and migrate to the draining lymph nodes under the influence of TNFα and IL-1β. LCs present processed antigen to naive T cells and induce immune responses that are either Th1 or Th2 types, depending on the cytokine environment. IL-12 promotes a Th1 responses whereas the IL-10 and IL-4 promote a Th2 response. Th2 response is associated with allergic reactions.

Tumor

Functional impairment of LCs in the skin is believed to increase the susceptibility of tumor development upon exposure to carcinogens, UV light, or oncogenic viral infections (e.g. papillomavirus)[33, 34]. In some cases, it is observed that presence of a large number of LCs in the tumor is of prognostic value[35, 36]. LCs are believed to contribute to the immune protection against tumors by directly secreting cytokines with anti-tumor activity and evoking systemic immune responses to tumor associated antigens (TAA).

Technologies that target LCs for immunization

Given the importance of APCs in the initiation and regulation of immune responses, a number of strategies and techniques have been examined in the past decade to employ LCs/DCs in the prophylactic and therapeutic immunizations against infectious diseases and immune disorders. Most technologies focus on the use of DCs because they can be generated *in vitro* from peripheral blood monocytes or bone marrow cells[37, 38]. The *ex vivo* DC-based vaccines are widely examined for cancer immunotherapy. This strategy involves *in vitro* generation of DCs, antigen loading, and transfusion of cells to the patient. In comparison, far fewer technologies that exploit epidermal LCs in immunization exist. The epidermis is too thin to be accessed by traditional syringe and needle injection. Application of vaccine antigens to the surface of an intact skin has been explored. Transcutaneous immunization (TCI) is a technology that topically delivers liquid vaccines using cholera toxin adjuvant[39]. Antibody responses were elicited to several protein antigens including diphtheria and tetanus toxoids, cholera toxin (CT), heat-labile toxin from E. coli (LT), and bovine serum albumin in mice[39]. TCI of humans with LT causes morphological changes in LCs, suggesting that LCs may play a role in the antibody response[40]. Topical immunization techniques may not be effective with large vaccine molecules or particle vaccine formulations (e.g. inactivated whole pathogens, or alum-adsorbed vaccines) because of the barrier function of the stratum corneum. Here we will focus discussion on a unique technology, Epi-

dermal Powder Immunization (EPI), which enables efficient delivery of vaccine antigens to the LC rich epidermis.

EPI is a novel technology that was developed to target antigens to LCs *in vivo*. EPI has its roots in a technology that was developed in the early1990s for genetically engineering plants and then was adapted for DNA immunization[41-43]. EPI delivers antigens in the form of microscopic particles to the epidermis using a needle-free powder delivery system (or PowderJect device) and elicits broad immune responses[44]. Here we will review our findings using this technology to deliver traditional vaccines in relation to LC targeting and its potential applications in the prevention and treatment of infectious diseases and immune disorders.

Many traditional vaccines such as proteins, peptides, polysaccharides, inactivated pathogens etc are suitable for EPI[44-47]. Vaccine powders can be prepared by coating antigens onto 1-2 μm gold particles or embedding them into 20-50 μm particles using sugar excipients (trehalose, mannitol, sucrose, or combinations)[44, 47]. The driving force of the device is pressured helium gas. Actuation of the device causes the release of helium gas, which accelerates the vaccine particles to high velocity that penetrate the stratum corneum and land in the LC rich viable epidermis. In addition to targeting antigen to the LC rich epidermis, EPI offers the advantage of pain-free delivery. This is because the sensory nerve endings in the epidermis are far less dense than deeper tissues such as dermis and muscle.

A. Evidence for targeting LCs

Histological data shows that EPI delivers powdered vaccines into the LC rich viable epidermis. Antigen targeting of LCs in the target tissue following EPI is dependent on whether gold or sugar formulation was used. EPI with the gold formulation results in intracellular deposition of both gold and antigen in the epidermal cells[47]. LCs account for about 3% of the total number of the epidermal cells (Fig 2A), thus, a fraction of particles is directly delivered to LCs. EPI delivers antigens to a skin target of approximately 110 mm^2, which comprises approximately 1×10^5 LCs[47]. Delivering a 0.5 mg of gold coated with antigen results in up to 50% of the LCs at the vaccination site containing antigen-coated particles as revealed by an immunofluorescence microscopy(47) (Fig 2B). The number of antigen-loaded LCs is sufficient to evoke an immune response.

EPI with a sugar formulation results in a different antigen distribution pattern than the gold formulation. The footprint of the sugar particles can be located by a tissue marking black dye, which binds to the cells with minimal diffusion after particle dissolution[48]. The discrete dark spots are visible under a light microscope (Fig 2C). Following sugar particle dissolution, the Tax Red labeled ovalbumin (TR-OVA) diffuses into the surrounding cells and appears to be picked up by LCs. Nearly all LCs in the target tissue appear to contain antigens (Fig 2D). Antigen and antigen carrying LCs are present at the site of immunization for up to 5 d although the concentrations are greatly reduced after the first 48 h (unpublished data).

EPI inherently activates LCs in the target site as evidenced by enlarged cell body and brighter staining with an I-A^d specific monoclonal antibody (enhanced expression of class II antigens)[48]. LC activation is probably mediated by cytokines produced by epidermal cells when triggered by the particle penetration. *In vitro* culture of epidermal sheets prepared from the immunization site result in the migration of LCs into the culture medium. *In vivo*, antigen-carrying LCs can be shown in the regional draining lymph nodes starting 20 h after the immunization and lasting for several days (Fig 2E). More recent studies indicate that transfer of LCs isolated from the immunization site to naïve mice induces antigen-specific antibody responses, confirming that LCs are the antigen presenting cells (unpublished data).

B. Breadth of the immune responses induced by EPI

B1. Serum antibody responses: EPI elicits strong serum antibody responses in mice to a variety of antigens including inactivated influenza viruses, diphtheria toxoid, hepatitis B surface antigen (HBsAg), and HIV gp120. Using the same vaccine, EPI elicited significantly higher serum titers than syringe and needle injection via common routes (e.g. subcutaneous, intramuscular, or intraperitoneal). Dose range studies indicate that EPI may require a con-

siderably smaller antigen dose than needle injection to achieve maximal immunity[44, 48]. The serum antibody titers are as long-lasting as those elicited by needle injection[45]. Recently, we showed that EPI elicits high serum antibody titers to influenza vaccine and HBsAg in monkeys, which have an immune system resembling that of humans.

B2. Mucosal antibodies. The majority of infectious agents gain entry to the human body through mucosal tissues, thus mucosal antibodies, i.e. secretory IgA (SIgA), in the mucosal surface are vitally important for protection. The immune system of humans and animals is compartmentalized in such a way that mucosal antibodies are only induced by direct application of vaccines to mucosal sites, whereas vaccination by a non-mucosal route (i.e. intramuscularly) normally does not elicit a mucosal immunity. However, recent data has shown that the skin immune system appears to cross talk with the mucosal immune system.

Following EPI of mice with an inactivated influenza vaccine, antigen-specific SIgA antibodies were detected in mucosal lavages of the small intestine, trachea, and vaginal tract[46]. The local origin of the SIgA antibodies was further shown by measuring antibodies released from cultured tracheal and small intestinal fragments and by detecting antigen-specific IgA secreting cells in the lamina propria using ELISPOT assays. These antibodies appear to be important for protection against experimental

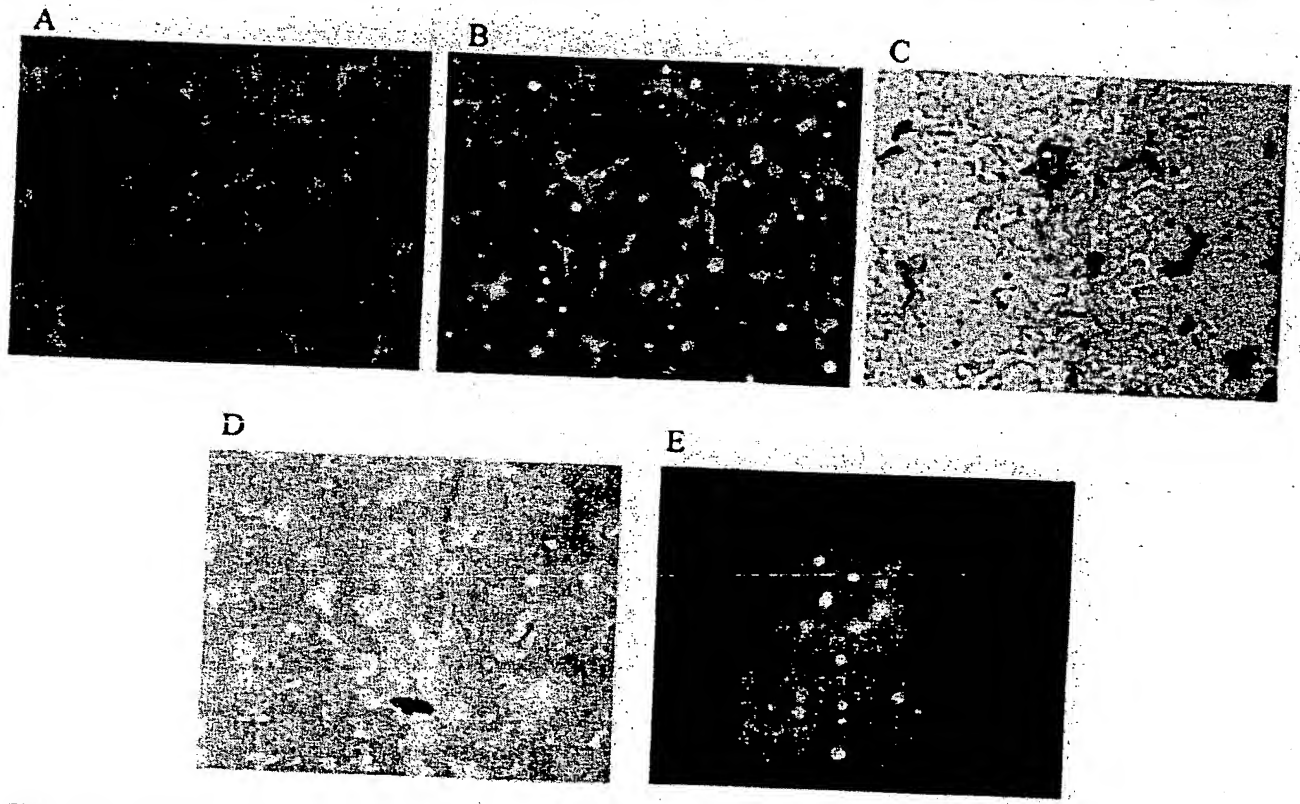

Fig 2. EPI targets antigens to LCs. A. Normal LCs (green) in the epidermal sheet stained using Mab to I-A^d antigen conjugated to fluorescein isothiocyanate (FITC) (60×). B. EPI using gold particles delivers antigen to the cytoplasm of LCs. EPI was performed using gold particles (1-2 μm) coated with TR-OVA (model antigen). Immediately after EPI, Epidermal sheet from the site of immunization was prepared and LCs were stained with Mab to I-A^d antigen conjugated to FITC. The gold particles inside the green-colored LCs are in yellow color, whereas the gold particles in non-fluorescent cells (keratinocytes) are in red color (60×). C. Epidermal sheet from the site of EPI using sugar particles (20-50 μm) containing TR-OVA and black tissue marking dye viewed under a light microscope (60×). Black spots indicate the footprint of vaccine particles. D. The same field shown in C viewed under an UV light. LCs (FITC-stained) become yellow once they take up antigen (60×) after the dissolution of vaccine-containing sugar particles. E. Migration of LCs from the vaccination site to the draining lymph node. The vaccination sites were treated with FITC (dissolved in DMF) by topical application 2 h prior to EPI with TR-OVA coated gold particles. Fluorescent cells in the draining lymph nodes were examined 20 h later by fluorescent microscopy under an UV light. The green cells are LCs that contain TR-OVA coated gold particles originating from the site of EPI (240×).

challenge. An adjuvant appears to be required for EPI to induce a mucosal antibody response. CT, LT, a synthetic oligodeoxynucleotide containing immunostimulatory CpG motifs (CpG DNA), and many other adjuvants were found to enhance the mucosal antibody responses to co-delivered antigens.

B3. Cytotoxic T lymphocyte response: CTLs play a vital role in host defense against viral and intracellular bacterial infections. However, non-replicating vaccines administered by intramuscular injection using a syringe and needle elicit predominantly humoral responses but not CTL responses. Proteins in the cytosol are processed and presented under the restriction of MHC class I molecules and induce CTL responses. EPI using gold particles delivers proteins to the cytosol of LCs and other epidermal cells. CTL responses were observed to HBsAg, HIV gp120, and an influenza virus nucleoprotein peptide in mice when loaded on to gold particles and administered by EPI[47, 48]. The CTL responses were similar to the responses elicited by DNA vaccines and live-attenuated vaccines. EPI with sugar powder formulations does not normally elicit a CTL response[44]. This is because antigens in the sugar formulation are delivered extracellularly. These antigens are taken up by LCs via phagocytosis and endocytosis.

B4. T helper responses and immunomodulation with adjuvants: The T helper cell responses (Th1 vs Th2) following EPI have been examined using a number of antigens. The T helper cell responses are dependent on the antigen dose and whether adjuvant is used. In the absence of an adjuvant, both diphtheria toxoid and HBsAg induce a Th2 response as reflected by the production of low level of IFN-γ and a high IgG1/IgG2a antibody ratio[45]. When decreasing antigen doses were tested, the Th2 response shifted toward the Th1 response (unpublished data). The T helper cell responses can be shifted towards Th1 or Th2 when appropriate adjuvants are used. For example, CpG DNA, QS-21 and LT adjuvants cause a shift to the Th1 response whereas the aluminum adjuvants and PCPP promote a Th2 response[45].

C. Potential applications

C1. Infectious diseases. EPI can elicit high levels of serum antibody, which are important for prophylactic immunization against infection by extracellular bacteria and many viruses. Mucosal antibodies are particularly important because these antibodies may prevent pathogens from gaining entry to the deeper tissue. EPI may have immunotherapy potential for chronic infections caused by viruses and intracellular bacteria since EPI with antigen coated gold particles elicit a CTL response, which may help to cure established infections by eliminating infected cells. It is also important to note that EPI may allow the use of new and more potent adjuvants in routine human vaccination, which otherwise may not be safe when administered into a deeper tissue. This is because adjuvants administered by EPI appear to be gradually released from the immunization site to deeper tissue over a period of days, thus reducing the peak serum concentration and systemic toxicity. Furthermore, periodic sloughing and regeneration of the epidermis remove any residual adjuvant in the skin that may cause chronic local reactions, a problem seen with deeper tissue injection.

C2. Cancer immunotherapy: Immunizations with whole tumor cells or lysates, and recently with tumor-associated (TAA) antigens by traditional methods have produced some promising clinical results[49]. Antigen presentation is a critical regulatory element for the induction of humoral and cellular immune responses to tumors. DCs are now being widely explored for immunotherapy against a variety of tumors. The commonly used technique for DC-based cancer immunotherapy involves ex vivo cultivation of autologous DCs from the peripheral blood cells, in vitro antigen loading, and the transfer of DCs to the patients.

EPI represents an *in vivo* technique for developing DC-based immunotherapy. It directly delivers antigens to the cytosol of the LCs and elicits both antibody and cellular immune responses. EPI is suitable for delivering tumor cell lysates, purified antigen, and other immunostimulating agents (e.g. GM-CSF). It is technically much more suitable for commercialization when compared with *ex vivo* DC-based approaches.

C3. Immunotherapy for allergic diseases. Allergic diseases including ACD, asthma, and hay fever are commonly treated with corticosteroids and antihistamine drugs. Immunotherapy by subcutaneous injection of allergen over a prolonged period has

been used to treat several types of allergies with variable clinical effects. It is effective in inducing prolonged remission of insect venom anaphylaxis, but is only moderately effective against hay fever, and even less effective against asthma. Although the mechanism of immune therapy in humans is not fully understood, studies using animal models suggest that immunization strategies promoting a Th1 response can decrease the formation of IgE antibodies and eosinophilia[50]. Alternatively, formation of IgG antibodies will neutralize the allergens before being captured by the IgE antibodies. EPI using antigen and an appropriate adjuvant (CpG DNA, saponin, etc) promoted strong Th1 responses in an animal model, suggesting that it may be possible to reprogram the immune system of the sensitized individual and offers a more effective means of allergy immunotherapy. Given the concept that the immune system is compartmentalized, administering treatment to skin by EPI may lead to maximal therapeutic effect in the skin and related mucosal tissues, which are normally the affected targets of allergies.

CONCLUSIONS

Given the central role of LCs and DCs in the initiation and regulation of the immune response to infectious pathogens, allergens, and tumor antigens, immunization strategies that target LCs/DCs may lead to more effective therapies against these diseases. Developing technologies for effective in vivo or in vitro targeting of LCs/DCs are ultimately important. EPI delivers antigen to the LC rich skin and generates robust immune responses in animal models. The quality of the immune responses (TH1 vs. TH2, mucosal vs. systemic, cellular vs. humoral) generated by EPI can potentially be tailored to specific therapeutic goals by choosing appropriate carrier particles and co-administration of an immunomodulator/adjuvant.

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Chapter 20

Water-Soluble Phosphazene Polymers for Parenteral and Mucosal Vaccine Delivery

Lendon G. Payne, Sharon A. Jenkins, Alexander Andrianov, and Bryan E. Roberts

1. INTRODUCTION

The advent of modern molecular biology has provided us with a means of producing antigens with unprecedented ease and precision. It is ironic that these new methodologies generate purified antigens that do not generally induce a strong immune response in the absence of an effective adjuvant. The development of improved vaccine adjuvants for use in humans has therefore become a priority area of research. Nevertheless, research on adjuvants has lagged seriously behind the work done on antigens. For decades the only adjuvant widely used in humans has been alum. Saponin and its purified component Quil A, complete Freund's adjuvant (CFA) and other adjuvants used in research and veterinary applications have toxicities that limit their potential use in human vaccines. New chemically defined preparations such as QS-21, muramyl dipeptide, and monophosphoryl lipid A are being studied.

The traditional view on how adjuvants exert their effect is that adjuvants such as mineral oil emulsions or aluminum hydroxide form an antigen depot at the site of injection that slowly releases antigen. However, excision of the injection site after only 3 days had little effect on immune responses (White, 1976). Recent studies indicate that adjuvants enhance the immune response by stimulating specific and sometimes very narrow arms of the immune response by the release of cytokines (Allison and Byars, 1992).

There is considerable interest in the development of controlled release vaccines, since the major disadvantage of several currently available vaccines is the need for repeated administrations. Controlled release vaccines could obviate the need for booster immunizations and would be particularly advantageous in developing countries, where repeated

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contact between the healthcare worker and the vaccine recipient is often difficult to achieve.

There is a growing body of evidence to suggest that antigen persisting on the external membrane of follicular dendritic cells and lymph node organs is involved in the recruitment of B memory cells to form antibody-secreting cells (Gray, 1993). The continual release of circulating antibodies suggests this recruitment happens continually. As the level of antigen decreases, affinity maturation of antibody occurs. This concept of antigen persistence has an important implication in vaccine development. Ideally, it would be advantageous to be able to formulate vaccines in a way such that antigen is presented to the immune system and in particular the follicular dendritic cells over an extended period of time.

An area of adjuvant research that has developed over the last few years is the utilization of synthetic polymers in formulating vaccines to effect the controlled release of antigens. The nonionic block copolymer surfactants (Hunter, 1991) with molecular weights below approximately 10,000 have a simple structure composed of two blocks of hydrophilic polyoxyethylene (POE) flanking a single block of hydrophobic polyoxypropylene (POP). They are considered to be among the least toxic of surfactants and are widely used in foods, drugs, and cosmetics. Some of the large hydrophobic copolymers are effective adjuvants, whereas closely related preparations are not. There is a correlation between the adjuvant activity of these copolymers with differences in the chain links of the POE and POP. Currently, these adjuvants are used in an oil and water emulsion.

A wide range of polyelectrolytes of various molecular weights have been shown to have adjuvant activity (Petrov *et al.*, 1992). Macromolecules bearing either positive or negative charges have displayed a similar immunostimulatory activity. The polyelectrolytes form complexes with antigens through electrostatic and hydrophobic bonds. On the other hand, neutral and uncharged polymers had no effect on the immune response unless the uncharged polymers were conjugated to the protein antigens.

Polymers have also been used to entrap antigens. An early example of this was the polymerization of methyl methacrylate into spheres having diameters less than 1 μm to form so-called nanoparticles (Kreuter, 1992; Chapter 19 this volume). The antibody response as well as the protection against infection with influenza virus was significantly better than influenza that was adjuvanted with aluminum hydroxide. Experiments with other particles demonstrated that the adjuvant effect of these polymers depends on particle size and hydrophobicity.

Microencapsulation has been applied to the injection of pharmaceuticals to give a controlled release. A frequent choice of a carrier for pharmaceuticals is poly D,L-lactide-co-glycolide (PLGA). This is a biodegradable polyester that has a long history of medical use in erodible sutures, bone plates, and other temporary prostheses. This widespread use of PLGA was achieved without any toxicity. The use of a biodegradable microencapsulation system that permits controlled release of antigens certainly presents a very attractive approach to mucosal immunization. In the last few years, a body of data has accumulated on the adaptation of PLGA for the controlled release of antigen (Eldridge *et al.*, 1989, 1991). The entrapment of antigens in PLGA microspheres of 1 to 10 μm in diameter has been shown to have a remarkable adjuvant effect. The disadvantage of the PLGA system is that the use of organic solvents and long preparation times for the microencapsulation

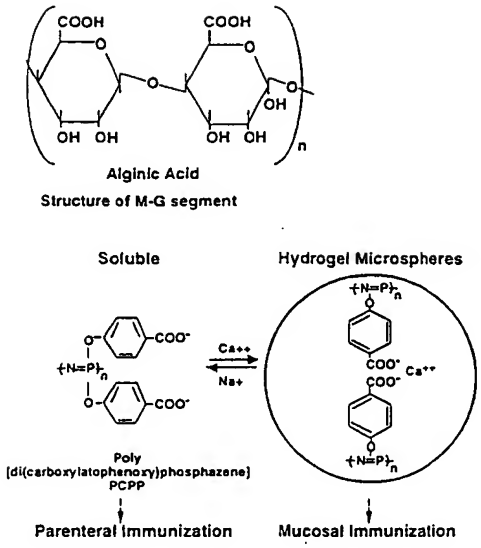

Figure 1. Ionically cross-linked polymers. The chemical structures for alginic acid and PCPP are shown in their un-ionized states. Ionic cross-linking is exemplified for the polyphosphazenes. The addition of heavy metal ions (e.g., Ca^{2+}) to the soluble polymer initiates cross-linking of polymer strands via the divalent cation, resulting in the formation of a hydrogel.

of the antigens may alter antigen conformation required for a functional or efficacious immune response.

The elucidation of a new class of ion cross-linkable water-soluble polyphosphazenes (PCPP) (Fig. 1) (Allcock and Kwon, 1989) has made it possible to generate microspheres in an aqueous environment (Cohen *et al.*, 1990; Payne *et al.*, 1994). The model for the development of the polyphosphazenes was the naturally occurring alginates prepared from brown algae and used in food stuffs. Gelation by ionic cross-linking of an aqueous-based polymer solution at room temperature eliminates the long exposure of antigens to organic solvents, elevated temperatures, and drying required by polymers dissolved in organic solvents. These characteristics make polyphosphazene microspheres an interesting potential vaccine delivery vehicle. Since anionic and cationic polymers have previously been shown to have immunoadjuvant activity, we were also interested in investigating the immunoadjuvant properties of the phosphazene and alginate polymers in the absence of ionic cross-linking. Thus, polyphosphazene can be combined with antigens in two different ways to potentially effect immunopotentiation. Antigens can be mixed with the soluble polyphosphazene and injected directly into an animal for parenteral immunization. Alternatively, the water-soluble polyphosphazene and antigen solution can be formulated into hydrogel microspheres by ionically cross-linking the carboxyl groups with divalent cations, and then used for parenteral or mucosal immunization.

Since the polyphosphazenes do represent a new class of polymers, it was of interest to determine the toxicity of this class of polymers. Cell culture dishes were coated with polyphosphazene and chicken embryo fibroblasts were seeded onto the coated petri dish. Three days after seeding, the cells had become flattened and spindlelike, and under contrast microscopy we could see mitotic figures. We have also encapsulated hybridoma cells in polyphosphazene microspheres having a diameter between 150 and 200 μm. The encapsulated hybridoma cells were able to undergo cell divisions, and by 10 days after

encapsulation the microspheres were essentially filled with living cells (Bano *et al.*, 1991). This demonstrated the innocuous nature of the polyphosphazenes in cell culture.

In vivo acute toxicity of alginate and polyphosphazene has been evaluated in 6- to 8-week-old Sprague–Dawley rats. The study consisted of four groups of five male rats/group. Following an overnight fast, each animal in each group received a single oral dose of 5000 mg/kg (in water) via gavage. The dose volume was 20 mL/kg. Group one rats were the control and received water. Group two animals received alginate microspheres. Group three rats received alginate microspheres coated with poly-L-lysine (PLL; 68 kDa) (Sigma, St. Louis, MO). Group four animals received polyphosphazene microspheres. The animals were clinically observed for 7 days. Body weights were recorded on day 1 prior to immunization and at euthanasia. Blood samples were obtained by puncture of the retroorbital sinus after anesthetization with CO_2 at euthanasia. Animals were faste overnight prior to blood collection. Tissues were examined and saved at necropsy. Ther were no significant differences in body weight gain between the rats that received microspheres and the rats in the control group. The results of hematology and clinical chemistry were normal for all rats in each group. There were no treatment-related abnormalities observed in any organ at necropsy. This study demonstrated that an oral dose of 5000 mg/kg of polyphosphazene or alginate microspheres was not acutely toxic.

2. MICROSPHERE FORMULATION AND CHARACTERIZATION

2.1. Microsphere Generation

Poly[di(carboxylatophenoxy)phosphazene] (PCPP) solutions were prepared by dissolving the appropriate amount of PCPP in one part 3% Na_2CO_3 while stirring, then slowly adding three parts phosphate buffer pH 7.4. The antigen solution was then slowly added to the polymer solution so that the final concentration of PCPP was 2.5%. Sodium alginate solutions were prepared by dissolving the appropriate amount of polymer in deionized water. The antigen was then slowly added to the alginate solution so that the final concentration of alginate is 1.25%. Constant stirring, as well as the slow addition of the antigen to the polymer, was necessary in order to obtain a homogeneous solution.

Protein molecular weight markers (Amersham) and FITC-labeled bovine serum albumin (BSA) (Sigma) have been microencapsulated to study release kinetics of soluble proteins. The release kinetics of 24-nm polystyrene beads (Duke Scientific) were also studied. Tetanus toxoid (TT) (Connaught Laboratories) and influenza virus were encapsulated for antigenicity studies. Influenza was grown in eggs according to standard methods and quantitated by protein, hemagglutination, and plaque assays. Influenza was inactivated by formalin by the addition of a 38% formaldehyde solution at a final dilution of 1:4000. Virus infectivity was also inactivated by exposure to gamma irradiation from a ^{60}Co source to 1.2×10^6 rad.

Previous work has shown that uptake of particulate material by Peyer's patch M-cells and subepithelial macrophages is limited to particles having diameters of 10 μm or less (Eldridge *et al.*, 1991). We therefore developed a process for generating microspheres in the size range of 1–10 μm (Fig. 2). The key component of our current encapsulation

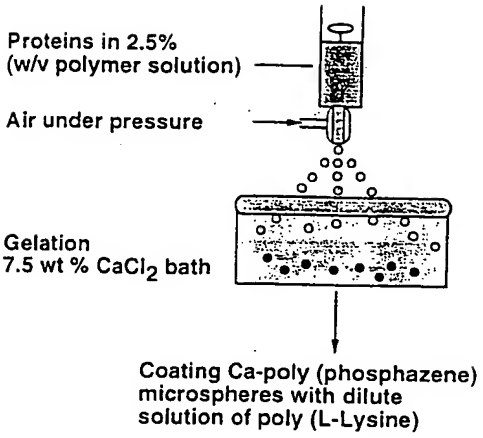

Figure 2. Microencapsulation process and size distribution. Cells or protein antigens are dispersed in an appropriate concentration of polymer to give a homogeneous solution. The solution is pumped into a Sonimist nozzle and forced through a 0.3-mm orifice by pressurized air, resulting in the generation of a microdroplet spray that impacts a calcium chloride bath. The polymer in the microdroplets is cross-linked (gelation) by the calcium ions to form microspheres. The microspheres can then be coated with other polymers.

procedure is an ultrasonic spray nozzle (Medsonic, Inc., Farmingdale, NY) that forces a polymer solution containing dispersed antigens through a small orifice under approximately 40 pounds per square inch of sterile air. The configuration of the nozzle results in the generation of a spray cloud containing microdroplets of polymer solution and antigen that impact a calcium chloride bath where the microdroplets begin to gel into microspheres. The microspheres are then collected and used as they are or can subsequently be coated with various other polymers such as PLL. Approximately 95% of alginate and PCPP microspheres generated under these conditions have diameters in the 1–10 μm range (Fig. 3). Ionically cross-linked microspheres were stored in buffers that are conducive to the maintenance of their integrity. Conditions were defined that maintain the integrity of the microspheres as well as the antigens entrapped within the polymer matrix. Microspheres containing antigen were stable for 7 days if stored at 4°C in sterile deionized water. Standard buffers such as phosphate-buffered saline (PBS) were not used because the replacement of calcium ions with sodium leads to the liquification of the matrix. Coating the microspheres with PLL allowed storage in PBS.

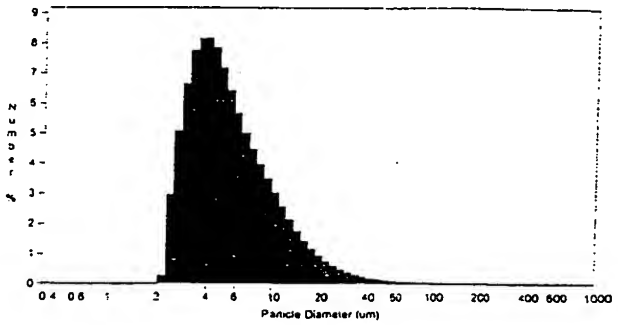

Figure 3. The size of the microspheres was analyzed by a Coulter LS 100 Particle Sizer. The graph shows the percentage of microspheres as a function of diameter.

2.2. Characterization of Microspheres

For immunogenicity studies, the protein content of microspheres was determined both directly after generation of the microspheres to assess the percent incorporation and also immediately before injection into animals to ensure delivery of known antigen quantities. The protein content of the microspheres could not be assessed by the Bio-Rad protein assay. Although the protein can be released from the microspheres by chelating the Ca^{2+} responsible for forming the hydrogel, the addition of the Bio-Rad reagent that contains divalent cations causes the polymer to re-cross-link rendering the antigen unavailable to the dye reagent. The quantitation of protein antigens encapsulated in ionically cross-linked microspheres was determined by electrophoresis of a known quantity of intact microspheres in SDS-PAGE. During electrophoresis, the proteins migrated out of the micro sphere matrix and into the polyacrylamide gel. The protein concentration was determined by comparison to known quantities of the encapsulated protein electrophoresed in parallel to the microsphere preparation.

A body of data accumulated on the adaptation of PLGA for controlled antigen release has shown that the entrapment of antigens in 1- to 10-μm microspheres has a remarkable adjuvant effect (Eldridge *et al.*, 1991). The size of alginate and PCPP microspheres was measured utilizing a Coulter LS100 Particle Sizer. The size is reported as % number in the size range 1–10 μm.

2.3. Antigen Release

In order for the microencapsulated antigens to elicit an immune response, the antigen must be released from the microspheres. Antigen is released from a microsphere through the two different but not mutually exclusive processes of diffusion and erosion (Fig. 4). If the hydrogel is permeable to the dispersed antigens, then the antigens can simply diffuse out of the microspheres following the water phase that fills the matrix of the microsphere. Release of antigen is, therefore, an indication of the permeability of the microsphere matrix

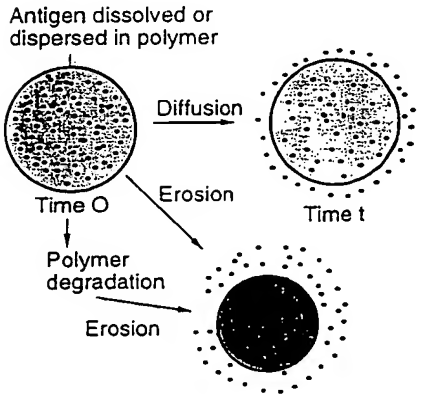

Figure 4. Release mechanisms of microencapsulated antigens. Polydispersed antigen can be released by simple diffusion or erosion. Erosion can occur through the redissolving of the polymer or by polymer degradation.

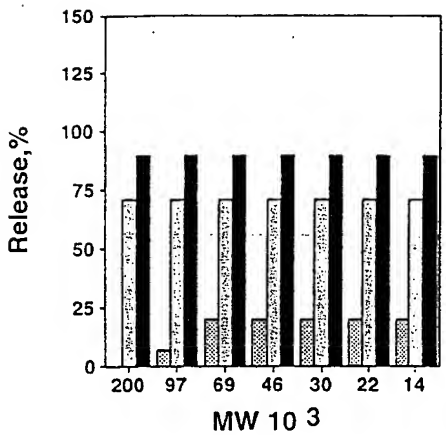

Figure 5. Permeability of polyphosphazene microspheres. Rainbow protein markers were microencapsulated in three concentrations of PCPP and incubated in HEPES buffer pH 7.4 at room temperature for 24 h before the amount of protein in the supernatant was spectrophotometrically measured.

to the antigen. Conversely, adsorption of the antigens to the polymer matrix will serve to either reduce or eliminate the diffusion of the antigen out of the microsphere.

The permeability of the PCPP microspheres was investigated by encapsulating the rainbow protein molecular weight markers (Amersham) that are commonly used in polyacrylamide gel electrophoresis (Fig. 5) (Andrianov *et al.*, 1993a). Release of the proteins was assayed by spectrophotometric measurements of the supernatant. The permeability of a particular protein such as the 14.3 kDa lysozyme was affected by the concentration of the polymer in the gel. As the polymer concentration rises from 1.5% to 3.3% there is a marked decrease in the diffusion of the protein out of the microsphere matrix. Similarly, as the molecular weight of the protein increases, diffusion of the protein out of the matrix is retarded. For example, the 200-kDa myoglobin protein was unable to diffuse out in a period of 24 h from a 3.3% PCPP matrix.

The second mechanism by which the antigens can be released from microspheres is through the erosion of the polymer matrix that makes up the microsphere (Fig. 4). Erosion occurs through the reversal of the gelation reaction, thus resulting in the solubilization of polymer molecules and their return to the surrounding aqueous environment. Degradation of PCPP microspheres was studied in saline solution (pH 7.4) by monitoring mass loss, molecular weights of polymer matrices and formation of soluble products. Erosion profiles for PCPP microspheres of varied molecular weights are shown in Fig. 6 (Andrianov *et al.*, 1993b, 1994). No detectable mass loss was observed during 180 days' incubation of high-molecular-weight PCPP microspheres in solution. However, gel permeation chromatography (GPC) showed significant decrease in polymer molecular weight during the same period of time (Fig. 7a). The mechanism of degradation apparently involves intramolecular carboxylic group catalysis. Use of low-molecular-weight PCPP for microsphere preparation leads to significant erosion of the hydrogel during the first 10 days and a decrease in molecular weight of polymer (Fig. 7b). Water-soluble polymeric products of practically the same molecular weight as in the matrix were detected. The data indicate that there is a molecular weight threshold of approximately 200 kDa in the release of PCPP from the matrix into the solution in this system. However, it is obvious that polymer solubility also

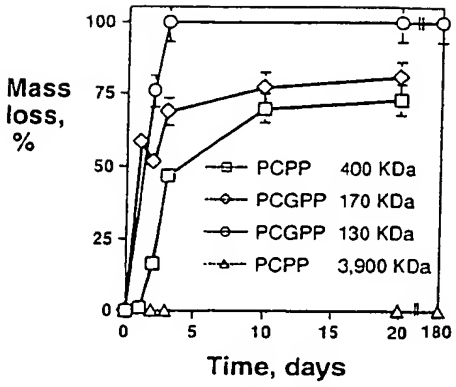

Figure 6. The effect of polymer side chains and molecular weight on microsphere erosion rates. Microspheres composed of PCPP and PCGPP were incubated in HEPES buffer pH 7.4 at 37°C. At various times, the dry weight of the microcapsules was assayed and expressed as the percent mass loss.

depends on the amount of calcium ions held by the matrix and the ionization degree of macromolecules.

Polyphosphazenes can be efficiently tailored by incorporating appropriate side groups to provide a controllable set of properties, including hydrolytic degradability. It was anticipated that introduction of a hydrolysis-sensitive pendant group, such as a glycinato group (Fig. 8), would result in an increased degradation rate in an aqueous environment. Cleavage of an external P–N bond occurring in neutral media in these aminophosphazenes to yield hydroxy derivative produces hydrolytic instability in the polymer. Poly[(carboxylatophenoxy)(glycinato) phosphazene] (PCGPP) containing 10% of glycinato groups was used for the preparation of microspheres and degradation studies. Erosion rates for these polymer hydrogels also depend on the molecular weight of PCPPs. PCGPP of weight

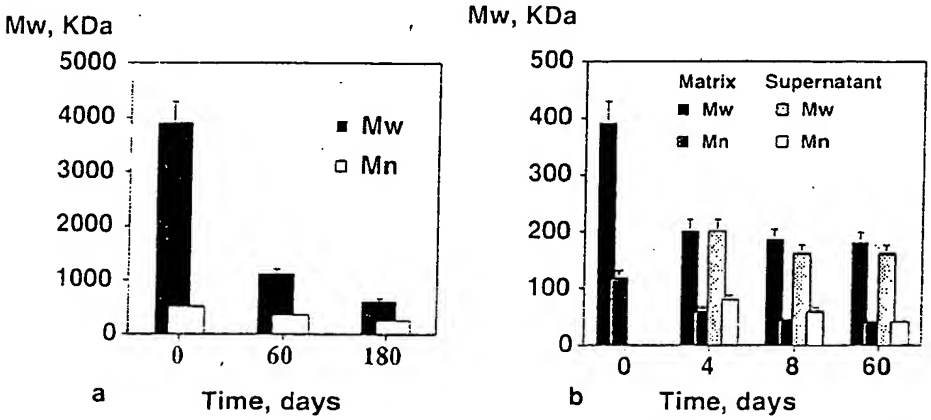

Figure 7. Molecular weight degradation profiles for PCPP hydrogel microspheres formed by ionic cross-linking of PCPP of 3900 kDa (a) and 400 kDa (b). Microspheres were incubated in HEPES-buffered saline (pH 7.4). Weight average and number average molecular weights of water-soluble degradation products in supernatant were determined. Matrix samples were isolated and then dissolved to measure weight average and number average molecular weights.

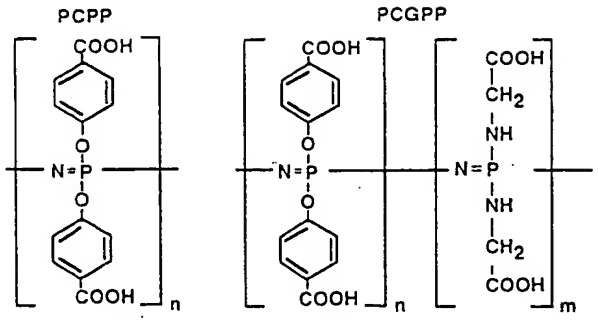

Figure 8. Chemical structures of PCPP and PCGPP. The ratio of the two side chains in PCGPP determines the rate of degradation.

average 130 kDa has a 100% mass loss within 3 days (Fig. 6). GPC analysis of matrix and soluble products (Fig. 9) showed that incubation for 240 days in an aqueous environment results in breakdown of the polymer backbone leading to fragments of less than 1 kDa and inorganic phosphate. Coating hydrogel microspheres with PLL (62 kDa) to yield a polyelectrolyte-complex membrane significantly decreases the erosion rate by 2.5 times because of steric hindrance. This appears to provide an additional approach to control the degradation and stability of PCPP microspheres. These results indicate the potential for specific regulation of the degradation kinetics and, therefore, release kinetics of encapsulated antigens. It is not known if this in vitro degradation mimics the in vivo situation, or if there is also enzymatically driven degradation.

The third means by which we have been able to regulate the release of antigen from microspheres is by coating the PCPP microspheres with PLL to form a semipermeable membrane on the outside of the microspheres (Fig. 10). The microsphere core can then be liquefied by the addition of chelating agents such as EDTA, which reverses the gelation process and results in the solubilization of the PCPP matrix. The degree of permeability can be regulated by the size of the PLL that is used in the coating process. We have used PLLs ranging from 12 to 295 kDa (Fig. 11). As the molecular weight of the PLL increases,

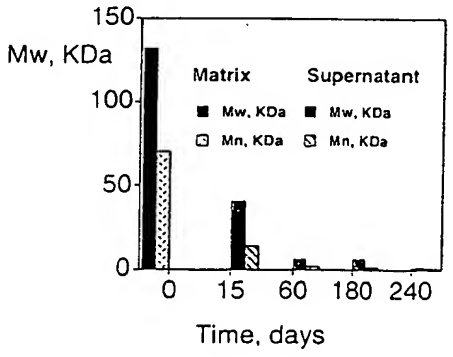

Figure 9. Molecular weight degradation profiles for PCGPP hydrogel microspheres formed by ionic cross-linking of PCGPP of 130 kDa. Weight average and number average molecular weights of water-soluble degradation products in supernatant were determined. Matrix samples were isolated and then dissolved to measure weight average and number average molecular weights.

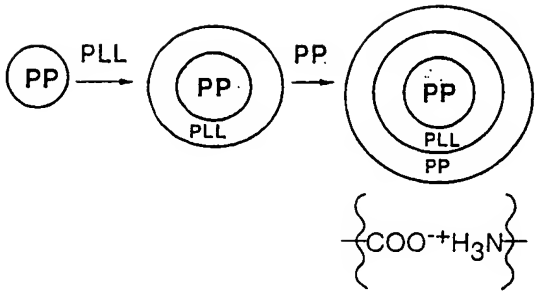

Figure 10. Coating of microspheres. Microspheres can be sequentially coated with polyanions. The positively charged poly-L-lysine (PLL) builds ionic linkages to the surface of the negatively charged polyphosphazene (PP) microspheres. Subsequently, another polymer coat can be added by reacting the PLL surface with negatively charged PP. In this way the surface charge and hydrophilicity of the microspheres can be regulated.

the permeability of the coating increases, resulting in an increased release of 24-nm polystyrene beads from the microsphere.

3. IMMUNOGENICITY STUDIES

3.1. Parenteral Immunization

Traditionally, most injected nonreplicating vaccines have required multiple doses to achieve protective serum antibody titers. For obvious reasons, it would be much more desirable to achieve protection with a single inoculation. Therefore, the effect of PCPP on the immunogenicity of antigens was examined in mice that were immunized subcutaneously with a single dose. Antigen formulated in water, alum, and CFA was included in many experiments for comparison.

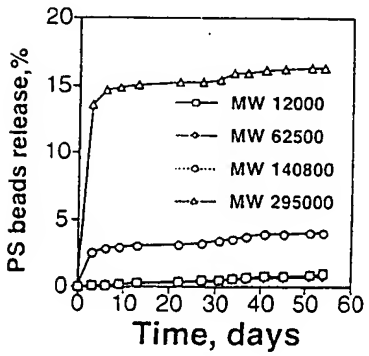

Figure 11. Release from microspheres coated with poly-L-lysines of different molecular weights. Fluorescent polystyrene (PS) beads measuring 20 nm in diameter were encapsulated in polymer 1 and then coated with poly-L-lysines of different molecular weights. The coated beads were incubated in HEPES buffer pH 7.4 at room temperature. Polystyrene beads released into the supernatant were measured by quantitative fluorimetry and expressed as a percent of the initially encapsulated beads.

Antigen was precipitated onto alum by adding dropwise 1.0 mL of a 10% solution of $Al(K)(SO_4)_2 \cdot 12H_2O$ (Sigma) to 2.5 mL of antigen solution with stirring, and adjusting the pH to 6.5 with approximately 0.65 mL of 1 N NaOH. After 30 min at room temperature, the suspension was centrifuged at 1000*g* at 5°C for 10 min. The amount of antigen adsorbed was determined by analyzing the amount of antigen in the supernatant and subtracting this amount from the total. The pellet was resuspended in sterile deionized water and adjusted to the proper concentration for injection. Antigens are also formulated into CFA (Sigma) by preparing an emulsion made with a 1:1 mixture of CFA and antigen in sterile deionized water.

Female 7- to 8-week-old BALB/c mice were randomized into groups. Mice were inoculated subcutaneously with a sample volume of 0.2 mL with a 25-gauge needle in the loose skin over the neck. Blood samples were taken from the retroorbital sinus of CO_2-anesthetized mice. Mice were euthanized with CO_2 in an inhalation chamber. It should be noted that all of the data reported here result from single dose immunizations.

Antibodies specific to TT in mouse serum were determined by a horseradish peroxidase ELISA in 96-well microtiter plates coated with 1 μg/mL TT. The end-point titers are the reciprocal of the greatest sample dilution producing a signal twofold greater than that of an antibody-negative sample at the same dilution. The ELISA for influenza antibody substitutes 10 μg/mL of influenza-infected MDCK cell lysate for TT in the coating step. The IgG1, 2a, 2b, and 3 isotypes of the ELISA-reactive TT and influenza-specific antibodies were determined by the detection of murine antibodies bound to the antigens. Influenza-specific functional antibodies were measured by 50% plaque reduction and hemagglutination inhibition assays (HAI). The HAI titers are expressed as the reciprocal of the highest dilution that completely inhibits hemagglutination of erythrocytes.

3.1.1. PCPP MICROSPHERES

The immunogenicity of TT antigen formulated in polymeric microspheres composed of alginate or PCPP was compared to soluble TT and TT in alum and CFA (Table I). Groups of five mice were immunized by the subcutaneous route with 20 μg of TT. The antibody response to TT was assayed by ELISA. Soluble TT antigen and alginate-microencapsulated TT induced a maximum titer of 512 by week 13. PCPP microspheres containing TT induced higher antibody titers at early times postimmunization than alum- or CFA-adjuvanted TT. Furthermore, PCPP microspheres containing TT induced antibody titers that were still rising at 13 weeks postimmunization. At this late time point, TT in PCPP microspheres had elicited a titer of 65,536, which was approximately 100-fold higher than seen for soluble TT and 2- to 4-fold higher than was seen for alum and CFA. PCPP microspheres were clearly superior to alginate microspheres in the induction of antibodies to TT.

The dose-dependent effect of immunization with TT was examined by immunizing mice with varying amounts of TT formulated into PCPP microspheres or CFA (Table II). The immunogenicity of TT in PCPP microspheres compared very favorably with TT formulated with CFA. At all time points and TT doses, the ELISA titers for the two formulations were within a fourfold dilution of each other.

Table I
Effect of Adjuvants on the Antibody Response to Tetanus Toxoid as Measured by ELISA[a]

	TT-specific titer at week				
	3	5	7	9	13
TT in water	<256	256	256	256	512
TT in alginate MS	256	512	512	512	512
TT in alum	2048	8192	16,384	32,768	32,768
TT in CFA	2048	16,384	16,384	32,768	16,384
TT in polyphosphazene MS	8192	16,384	32,768	32,768	65,536

[a]Mice were immunized s.c. with 20 μg of tetanus toxoid (TT).

3.1.2. SOLUBLE PCPP

The ability of PCPP microspheres to dramatically potentiate the immune response to TT raised the possibility that the polymer could be acting as an immunoadjuvant, rather than as a simple vaccine vehicle. To examine this possibility, TT was mixed in an aqueous solution of PCPP but the antigen polymer solution was not formulated into microspheres (Table III). The antigen polymer solution elicited, in a dose-dependent manner, antibodies to TT. PCPP at 0.5% enhanced the immune response to TT more than 100-fold compared to the response to TT in water. PCPP at 0.05 and 0.005% concentrations also elicited higher antibody titers than TT in water, although not as high as what was obtained with 0.5% PCPP. Furthermore, the 0.5% PCPP concentration was as strong an adjuvant as CFA.

We next compared the effect of various TT antigen doses formulated in 0.1% PCPP solutions with 25 μg TT formulated in either water or CFA (Table IV). As expected, there was a clear antigen dose-dependent response at all time points using soluble PCPP. The 25 μg TT dose formulated into 0.1% PCPP elicited antibody titers that were dramatically higher than the same amount of immunogen in water, and compared very favorably with 25 μg of TT in CFA. It should be noted that at the 5, 1, and 0.2 μg immunogen dose levels

Table II
Effect of Encapsulated Tetanus Toxoid Dose on the Antibody Response to Tetanus Toxoid as Measured by ELISA

	TT-specific titer at week							
	PCPP microspheres				CFA			
TT (μg)	3	5	7	9	3	5	7	9
25	32,768	65,536	131,072	131,072	16,384	131,072	262,144	262,144
5	8192	32,768	65,536	65,536	4096[a]	16,384[a]	32,768[a]	16,384[a]
1	4096	16,384	65,636	65,536	16,384	32,768	32,768	32,768
0.2	2048	4096	8192	8192	1024	4096	4096	4096
0.04	<256	<256	256	256	<256	<256	<256	<256

[a]Mice were immunized s.c. with 2.5 μg tetanus toxoid (TT).

Table III
Effect of PCPP Concentration on the Antibody Response to Tetanus Toxoid as Measured by ELISA[a]

	TT-specific titer at week			
	3	5	7	9
TT in water	1024	2048	2048	4096
TT/0.5% PCPP	65,536	262,144	524,288	524,288
TT/0.05% PCPP	16,384	32,768	32,768	65,636
TT/0.005% PCPP	4096	8192	32,768	32,768
TT/CFA	16,384	131,072	262,144	262,144

[a]Mice were immunized s.c. with 25 μg of tetanus toxoid (TT).

in PCPP, the antibody titers were still rising at week 25, whereas with the CFA formulation the ELISA titers had peaked earlier.

Mice were also immunized with 5 μg of formalin-inactivated influenza virus particles formulated in polymeric microspheres, alum, and CFA to determine if the relative efficiencies of the formulations would be the same for an enveloped virus as they were for TT (Table V). Again, PCPP microspheres were as efficient as CFA but much more efficient than water, alum, or alginate microspheres at inducing a very high titer anti-influenza immune response. In contrast to the TT results, alum-adjuvanted influenza was no better than soluble influenza and alginate-microencapsulated influenza in eliciting a rather low titer anti-influenza response. Taken together, these results demonstrate that PCPP microspheres containing an immunogen provoke an antibody response equal in magnitude to immunogens formulated with CFA.

The mouse sera were also tested for the presence of functional antibodies by hemagglutination inhibition (Table VI) and neutralization assays (data not shown). As measured by the HAI assay, the PCPP microspheres containing influenza elicited an antibody titer of 1280 by week 7. Influenza formulated in water, alum, CFA, and alginate microspheres elicited either no detectable or very low HAI titers. Antibodies that neutralize influenza

Table IV
Effect of PCPP-Adjuvanted Tetanus Toxoid Dose on the Antibody Response as Measured by ELISA[a]

	TT-specific titer at week				
	3	6	9	17	25
25 μg TT/0.1% PCPP	16,384	65,536	131,072	>524,288	262,144
5 μg TT/0.1% PCPP	4096	16,384	32,768	65,536	131,072
1 μg TT/0.1% PCPP	2048	16,384	16,384	32,768	65,536
0.2 μg TT/0.1% PCPP	512	1024	1024	2048	4096
25 μg TT in water	2048	2048	8192	8192	16,384
25 μg TT in CFA	16,384	131,072	262,144	131,072	131,072

[a]Mice were immunized s.c.

Table V
Effect of Adjuvants on the Antibody Response to Influenza as Measured by ELISA[a]

	Flu-specific titer at week				
	3	5	7	9	13
Flu in water	256	1024	1024	512	512
Flu in alginate MS	512	1024	2048	2048	2048
Flu in alum	<256	512	1024	2048	2048
Flu in CFA	8192	16,384	32,768	32,768	16,384
Flu in polyphosphazene MS	8192	32,768	32,768	8192	16,384

[a]Mice were immunized with 5 μg of whole formalin-inactivated influenza virus particles.

infectivity were assayed in a 50% plaque reduction assay. Influenza in PCPP microspheres induced a detectable titer of 800 by week 13, whereas influenza in water and CFA did not elicit detectable neutralizing antibody titers. This result was very encouraging since the HAI and neutralization assays are sensitive functional antibody assays for influenza.

The IgG isotypes of the antibodies induced by these formulations were determined by an ELISA assay (Table VII). Alum-adjuvanted influenza elicited a purely IgG1 response as was expected. Influenza formulated in CFA induced mostly an IgG1 response that peaked by week 7 and waned by week 13. Influenza formulated in alginate and PCPP microspheres also induced largely an IgG1 response that by week 7 was higher than influenza formulated in alum. Again, PCPP microsphere-formulated antigen induced titers that compared very favorably with those induced by CFA-formulated antigen. PCPP microspheres, like CFA, were able to induce significant levels of IgG2a and IgG2b antibodies. A significant difference in the immune response was found in the level of activity detected in the IgG3 isotype. PCPP microspheres were the only formulation able to induce a significant IgG3 antibody titer.

Table VI
Effect of Adjuvants on the Antibody Response to Flu Proteins as Measured by the Influenza Hemagglutination Inhibition Assay[a]

	HAI titer at week				
	3	5	7	9	13
Flu in water	neg	neg	neg	40	neg
Flu in alginate MS	neg	neg	40	40	40
Flu in alum	neg	neg	neg	neg	neg
Flu in CFA	neg	neg	neg	40	neg
Flu in polyphosphazene MS	320	640	1280	1280	1280
Water[b]	neg	neg	neg	neg	neg

[a]Mice were immunized s.c. with 5 μg of whole formalin-inactivated influenza virus particles.
[b]Negative control had a titer of 20 because of nonspecific serum hemagglutination inhibitors. Neg $\leq$ 20.

Table VII
Effect of Adjuvants on the Antibody Isotype Response to Influenza as Measured by ELISA[a]

	Antibody isotype titer at week											
	3				7				13			
Adjuvant Used	IgG1	IgG2a	IgG2b	IgG3	IgG1	IgG2a	IgG2b	IgG3	IgG1	IgG2a	IgG2b	IgG3
Flu in alginate MS	1024	<256	256	<256	65,536	1024	512	<256	8192	512	<256	<256
Flu in PCPP MS	8192	4096	512	512	131,072	16,384	1024	4096	16,384	16,384	2048	1024
Flu in alum	512	<256	<256	<256	16,384	<256	<256	<256	8192	<256	<256	<256
Flu in CFA	8192	1024	4096	<256	>524,288	8192	4096	<256	32,768	2048	2048	<256
Flu in water	256	512	256	<256	2048	1024	256	<256	1024	512	<256	<256

[a]Mice were immunized with 5 μg of whole inactivated influenza virus particles.

Table VIII
Effect of PCPP-Adjuvanted Influenza Dose on the Antibody Response to Influenza as Measured by ELISA[a]

	Flu-specific titer at week					
	3	6	9	17	25	37
5 μg flu/0.1% PCPP	2048	16,384	16,384	32,768	65,536	16,384
1 μg flu/0.1% PCPP	4096	16,384	16,384	21,768	131,072	16,384
0.2 μg flu/0.1% PCPP	<256	4096	4096	16,384	65,536	8192
0.04 μg flu/0.1% PCPP	<256	<256	<256	<256	4096	1024
5 μg flu in water	256	256	256	<256	<256	<256
5 μg flu in CFA	512	4096	4096	2048	1024	2048

[a]Mice were immunized s.c.

The successful immunopotentiation of the influenza-specific immune response with immunogen formulated in PCPP microspheres prompted us to examine the possibility that influenza could also be adjuvanted with soluble PCPP. Mice were inoculated subcutaneously with varying influenza doses formulated in 0.1% PCPP or 5 μg of influenza in water or CFA (Table VIII). As expected, there was a dose-dependent immune response at all time points after inoculation with the 0.1% PCPP-formulated influenza antigen. In this experiment, 5 μg of influenza in 0.1% PCPP induced a dramatically higher influenza-specific response than 5 μg of influenza in CFA. Furthermore, all antigen doses in the PCPP formulation elicited an immune response that was still rising at week 25 whereas the CFA formulation induced peak titers at earlier time points. This was similar to the results seen in the TT experiments. It is particularly noteworthy that the 0.04 μg dose in PCPP did not induce detectable antibody levels until week 25. This can be interpreted as evidence for sustained release of antigen. The ability of 0.1% PCPP solution to induce functional antibodies was assayed in HAI (Table IX) and neutralization (Table X) assays. Once again, the PCPP formulation induced very high antibody activities in the hemagglutination and

Table IX
Effect of Influenza Dose on the Antibody Response to Flu Proteins as Measured by the Influenza Hemagglutination Inhibition Assay[a]

	HAI titer at week					
	3	6	9	17	25	37
5 μg PCPP	160	1280	1280	2560	640	1280
1 μg flu/0.1% PCPP	320	1280	1280	2560	2560	1280
0.2 μg flu/0.1% PCPP	40	640	640	2560	1280	1280
0.04 μg flu/0.1% PCPP	neg	40	80	160	160	160
5 μg flu in water	neg	neg	20	neg	neg	neg
5 μg flu in CFA	neg	80	40	40	40	40

[a]Mice were immunized s.c.

Table X
Effect of PCPP Concentration on the Antibody Response to Flu Proteins Measured by the Influenza Neutralization Assay[a]

	50% plaque reduction titer at week					
	3	6	9	17	25	37
5 μg flu/0.1% PCPP	200	400	400	200	400	1600
1 μg flu/0.1% PCPP	400	—	200	400	400	400
0.2 μg flu/0.1% PCPP	<100	—	100	100	400	400
0.04 μg flu/0.1% PCPP	<100	—	<100	<100	—	<100
5 μg flu in water	<100	<100	<100	<100	—	<100
5 μg flu in CFA	<100	<100	<100	<100	—	<100

[a]Mice were immunized s.c.

neutralization assays whereas there was little or no activity detectable in these assays in CFA formulations.

The influenza vaccine is administered to humans without alum because alum has little positive effect on the immune response. In a mouse potency test, an antigen dose that induces HAI antibody titers ≥40 units is predictive of protection in a human. Thus, 0.04 μg of total influenza antigen in 0.1% PCPP was able to induce protective levels of antibody that were not achieved with 5 μg of unadjuvanted antigen.

The antibody isotypes engendered in this response were also assayed (Table XI). Although the PCPP-formulated influenza antigen induced largely an IgG1 response, significant IgG2a and IgG2b responses were also detected. The level of this response was greater than that observed for influenza antigens formulated in CFA. In contrast to the results observed in Table VII, no IgG3 antibodies were detectable in this experiment. It should be noted, although not overinterpreted, that the IgG3 antibody responses were observed with influenza antigen formulated into PCPP microspheres but not in soluble PCPP solutions. Further experimentation must be conducted in order to confirm these results.

Table XI
Effect of PCPP-Adjuvanted Influenza Dose on the Antibody Isotype Response to Influenza as Measured by ELISA[a]

	Antibody isotype titer at week								
	3			6			9		
	IgG1	IgG2a	IgG2b	IgG1	IgG2a	IgG2b	IgG1	IgG2a	IgG2b
5 μg flu/0.1% PCPP	8192	<256	<256	131,072	<256	256	131,072	256	1024
1 μg flu/0.1% PCPP	8192	<256	<256	65,536	256	1024	65,536	1024	4096
0.2 μg flu/0.1% PCPP	256	<256	<256	16,384	1024	2048	16,384	1024	1024
5 μg flu/water	<256	1024	<256	256	1024	<256	256	512	<256
5 μg flu/CFA	2048	<256	<256	16,384	1024	512	16,384	1024	256

[a]All samples had IgG3 antibody titers <256.

All of the above experiments have been conducted using PCPP having only a phenoxycarboxy side chain, or PCPP. More recent experimentation has been done to examine the immunopotentiation effect of PCGPP (data not shown). In addition to the phenoxycarboxy side chain, PCGPP has a glycine side chain at a 10% frequency. PCGPP immunopotentiates the immune response to influenza virus compared to influenza in water, but is unable to increase the immune response to the level achieved with antigen formulated in PCPP. We are currently trying to determine the molecular basis for this difference in immunopotentiation.

3.2. Mucosal Immunization

An alternative to the use of injectable vaccines is the mucosal administration of a live attenuated virus. Such a vaccine induces both a strong oral and systemic immunity mimicking the immune response induced by natural infection with the wild-type virus. This constellation of immune responses eliminates not only the systemic spread of virus but also viral replication in the mucosa. Thus, the immune response elicited by a replicating oral vaccine is superior to that induced by injectable vaccines, either live or inactivated. The best example of this type of vaccine is the live attenuated oral poliovirus vaccine. Unfortunately the use of this vaccine is associated with reversion to neurovirulence and the development of paralytic polio in a few vaccinees and their contacts. The increasing appreciation of the importance of mucosal immunity in protection has spawned in recent years efforts to induce both the mucosal and systemic immunity by the delivery of nonreplicating viral antigens to mucosal surfaces.

Vaccines formulated into microspheres can be used to deliver antigen to the respiratory tract in the form of an aerosol or intranasal droplets. This delivery mode induces a mucosal response in lymphoid tissue located in the nasal pharynx and bronchial mucosa. Microsphere vaccines can also be formulated into a tablet or solution format that can be swallowed. This delivery mode induces a mucosal response in lymphoid tissue located in the pharynx and intestinal mucosa. Regardless of which site is the target for initiating the mucosal response, the existence of the common mucosal system results in specific IgA secretion at all mucosal sites.

The most studied ion-cross-linkable polymer is the naturally occurring alginate that is prepared from brown algae for use in foodstuffs. Although we use alginates in our microencapsulation of immunogens, most of the present in vitro data pertain to the synthetic ion-cross-linkable PCPPs. Alginates and PCPPs are cross-linked by di- and trivalent metallic ions to form hydrogels. The extent of cross-linking and, therefore, the rigidity of the prepared microspheres is influenced by polymer molecular weight, concentration, and cation concentration.

Microencapsulated antigens were prepared and quantitated as described above. The antigen concentration in alginate and PCPP microspheres as determined by SDS-PAGE was adjusted with sterile deionized water before immunization. Female 7- to 8-week-old BALB/c mice were randomized into groups and immunized by intranasal instillation with 48 μL of the antigen formulations. Blood samples were taken from the retroorbital sinus of CO_2-anesthetized mice. The blood was centrifuged and sera collected and stored at −70°C until it was analyzed.

Table XII
Effect of Adjuvants on the Antibody Response to Tetanus Toxoid after a Single Intranasal Immunization[a]

Mouse No.	Antigen formulation	Serum TT-specific titer at week						
		2	4	8	13	17	21	28
1	TT in water	256	<256	256	<256	<256	<256	<256
2	TT in water	<256	<256	<256	512	<256	<256	<256
3	TT in water	<256	<256	<256	256	<256	<256	<256
4	TT in Alg MS	<256	<256	<256	<256	<256	<256	<256
5	TT in Alg MS	<256	<256	<256	<256	<256	<256	<256
6	TT in Alg MS	<256	<256	<256	<256	<256	<256	<256
7	TT in PCPP MS	1024	1024	1024	2048	256	1024	1024
8	TT in PCPP MS	1024	1024	4096	4096	2048	8192	16,384
9	TT in PCPP MS	512	1024	2048	1024	1024	2048	2048
10	TT in Alg/5% PCPP MS	2048	8192	32,768	16,384	4096	8192	31,768
11	TT in Alg/5% PCPP MS	2048	4096	8192	16,384	4096	32,768	65,536
12	TT in Alg/5% PCPP MS	1024	2048	4096	4096	4096	2048	8192

[a]Mice were immunized by the intranasal instillation of a single dose of 50 μg tetanus toxoid (TT) in 48 μL.

The immunogenicity of a single 50 μg dose of TT (Table XII) was tested in four different formulations; (1) in water, (2) in alginate microspheres, (3) in PCPP microspheres, and (4) in microspheres composed of 95% alginate and 5% PCPP. Water-based formulations and alginate microspheres were unable to elicit serum IgG responses after intranasal instillation of mice. On the other hand, PCPP microspheres and alginate/PCPP microspheres induced very strong long-lived serum IgG responses to TT. Mucosal IgA specific for TT was not detected at any time point in the bronchial and lung washes of any mice. There was, however, a distinct, albeit transient, appearance of TT-specific IgA in the serum between weeks 2 and 8 postimmunization (data not shown). In a dose–response experiment, a 50 μg dose in microspheres composed of 95% alginate and 5% PCPP elicited a serum IgG response whereas a 5 μg dose did not (data not shown).

3.3. The Role of Polyphosphazene in Vaccine Delivery

Polyphosphazene is a water-based ionically cross-linkable polymer that can be used to generate polymeric hydrogel microspheres. Encapsulation conditions often play an important role in maintaining immunogenic integrity of labile antigens. Because there are no organic solvents involved in the encapsulation procedure, labile immunological epitopes are most likely preserved. Mild encapsulation conditions, hydrophobic surface properties, ability to alter the side chains on the polymer and formulate microspheres that will release antigens with pulsatile and/or sustained release kinetics make this polymer system a strong candidate for developing single-dose parenteral and mucosal vaccines.

Polyphosphazene microspheres have three desirable properties. Microspheres have the size and physical characteristics that facilitate uptake in the mucosal lymphoid tissue to stimulate an immune response. The microspheres are formulated under very mild

conditions so that antigenic integrity is maintained. The hydrogel properties of these microspheres allow a sustained antigen release to maximally stimulate the immune response over a long period. The microsphere technology has demonstrated great utility in the microencapsulation of a diverse spectrum of biological materials.

The immunogenicity of influenza and TT is dramatically enhanced in the presence of PCPP as either microencapsulated or soluble PCPP formulations. The results of these experiments clearly demonstrate that soluble PCPPs at very low concentrations are as efficient as PCPP microencapsulation and CFA at inducing high serum IgG responses. Furthermore, influenza admixed with soluble PCPPs or encapsulated in PCPP microspheres elicited HAI and neutralization antibody titers that were much higher than what was observed for alum and CFA.

Polyphosphazene, in either its soluble state or as microspheres, has two important characteristics in its interaction with antigens: sustained antigen release and the maintenance of antigenic integrity. Sustained antigen release has the benefit of potentially inducing longer-lasting immunity and the conversion of nonresponders. The data also suggest that smaller immunogen doses are required with this adjuvant, which lowers production costs. The antigenic integrity is evident in the induction of high titers of functional antibodies. Sustained antigen release and maintenance of antigenic integrity are characteristics of an immunoadjuvant that may have the greatest effect on antigens that are poorly immunogenic. Polyphosphazene has, thus, been proven to be a versatile molecule with both adjuvant and antigen depot characteristics.

4. SUMMARY

PCPP can be used in two different ways to potentiate an immune response. The soluble form of the polymer has been found to have immunoadjuvant activity. A single subcutaneous injection of polymer/influenza dramatically increases the ELISA, neutralizing, and HI antibodies to influenza virus compared to CFA. The polymer has also succeeded in dramatically increasing the amount of ELISA antibodies to TT. The antibody response elicited was predominantly of the IgG1 isotype. PCPP has also been used to generate micron-sized hydrogel microspheres through a process of divalent ion cross-linking of the polymer strands. These microspheres can induce significantly higher anti-TT serum IgG titers after a single intranasal immunization than TT alone.

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Utility of SHIV for Testing HIV-1 Vaccine Candidates in Macaques

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Summary: Intravenous injection of SHIV (simian/human immunodeficiency virus, chimeric virus) into rhesus macaques resulted in a viremia in peripheral blood lymphocytes (PBL) and the generation of anti-HIV-1 (human immunodeficiency virus type 1) envelope immune responses. A challenge stock of a SHIV containing HIV-1 HXBc2 envelope glycoproteins was prepared from infected rhesus monkey peripheral blood mononuclear cells (PBMC). The minimum animal infectious dose of the SHIV stock was determined and used in a challenge experiment to test protection. The vaccination of two rhesus monkeys with whole inactivated HIV-1 plus polydicarboxylatophenoxy phosphazene (PCPP) as the adjuvant protected the animals from becoming infected by a SHIV challenge. This experiment demonstrated for the first time that monkeys immunized with HIV-1 antigens can be protected against an HIV-1 envelope-containing virus. As the challenge virus was prepared from monkey PBMC, human antigens were unlikely to be involved in the protection. Protection of rhesus monkeys from SHIV challenge may help define protective immune responses stimulated by HIV-1 vaccine candidates. Key Words: Simian/human immunodeficiency virus chimera—Rhesus monkeys—HIV-1 vaccine.

One of the obstacles to HIV-1 vaccine development is the lack of an applicable animal model. The only nonhuman host in which HIV-1 can efficiently replicate is a chimpanzee. However, the cost and availability of this endangered species make it almost impossible to do sufficient experimentation needed to test vaccine efficacy. Although SIV (simian immunodeficiency virus) causes an AIDS-like disease in monkeys and its genetic organization shares remarkable similarity with HIV-1, vaccine studies have indicated that the immune reactive epitopes of the SIV envelope glycoprotein are different from those in its HIV-1 counterpart (1). This critical difference in the major protective antigen may diminish the predictive value of SIV as a model for HIV-1 vaccine candidates.

To overcome this problem, a molecular hybrid of SIV and HIV-1, designated SHIV, was engineered by replacing the *env*, *tat*, and *rev* genes of SIVmac239 with their respective HIV-1 HXBc2 counterparts. The SHIV-HXB construct used in this study also contains the HIV-1 *vpu* gene, which is

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absent in all known SIV genomes and whose expression in SHIV appears to enhance viral replication in vivo (20). This hybrid virus combines SIV's replicative ability in macaques monkeys together with the ability to encode HIV-1 envelope proteins. It has been shown that SHIV replicated in macaques cynomologus with a detectable viremia that lasted at least 3 months. All SHIV-infected monkeys had antibodies against the HIV-1 envelope glycoprotein, and many animals also produced antibodies reactive with the SIV gag proteins (2). These observations indicate the SHIV infection of macaques may serve as a valid animal model for assessing the efficacy of HIV-1 envelope-based vaccines.

MATERIALS AND METHODS

Preparation of the SHIV Challenge Stock

Approximately 100 ml of venous blood was collected from eight rhesus monkeys housed at the New England Regional Primate Research Center. Peripheral blood mononuclear cells (PBMC) were isolated by histopaque layer centrifugation and stimulated in culture by Concanavalin A (Con-A). The cells were then infected with SHIV-HXB that was prepared from infected CEM×174 cells and contained an reverse transcriptase (RT) activity of 10,000 cpm (2). The infected monkey PBMC were cultured in RPMI 1640 containing 10% fetal calf serum (FCS) and 20 U/ml recombinant human IL-2 for 5 days. The supernatant of the infected monkey PBMC was collected at days 5 and 6 postinfection (p.i.) and was filtered through a 0.45-μm filter unit. This culture supernatant consisted of an ~800 ml volume and was aliquotted into 1-ml cryovials and stored at −80°C. A number of the frozen vials were tested for $TCID_{50}$ (median tissue culture infectious dose) using CEM×174 cells. The radiolabeled cell lysates were precipitated with pooled sera from HIV-1–infected individuals to document the hybrid nature of the SHIV virus. Proteins corresponding to the HIV-1 *env* and *vpu* products and to the SIVmac *gag/pol* products were identified (data not shown).

Detection of SHIV Replication in *Macaca mulatta*

The infectivity of the SHIV challenge stock was tested in rhesus monkeys by means of intravenous injection. Approximately 10 ml of heparinized blood was collected from each inoculated animal at various times p.i.. PBMC were isolated by histopaque layer centrifugation and washed with phosphate-buffered saline (PBS). Approximately 4×10^6 monkey PBMC were co-cultivated with 2×10^6 CEM×174 cells in RPMI 1640 media containing 10% FCS. The mixed cells were stimulated with Con-A (5 μg/ml) overnight and were then cultured in RPMI 1640 medium containing 20 U/ml human IL-2. The detection of SIV gag p27 released into the culture medium by the SHIV infected cells was assayed a total of three times at 2-, 3-, and 4-week intervals following co-cultivation using Coulter's SIV core antigen detection kit (Coulter, Hialeah, FL, U.S.A.). Virus detection was scored as positive when p27 was present in the culture medium in at least two of the three time points. Virus load in the PBMC was determined by limiting dilution co-culture. In brief, 12 serial 1:3 dilutions of PBMC beginning with 1×10^6 cells were co-cultured in duplicate with 1×10^5 CEM×174 cells per well in 24 well plates (total volume, 1 ml). After 3–4 days of culture, 1 ml of RPMI 1640 media was added to each well. The culture was then split every 3 days at a 1:1 dilution. Supernatant samples were collected after 21 days of culture and assayed for SIV p27 antigen. Virus load was calculated as the number of PBMC present in the dilution at which 50% of the co-cultures were infected as determined by the p27 assay.

Serum was separated from clotted blood samples by low-speed centrifugation. The presence of anti-SHIV antibodies in the serum was tested by Western analysis using Dupont HIV-1 strips. $CD4^+$ T-cells were measured as described previously by Daniel et al. (3).

HIV-1 gp120 Antibody ELISA, Neutralizing Antibody Assay, and PCR Analysis

Recombinant HIV-1 gp120 (HXBc2 strain) was produced from a baculovirus expression system and was purified by either lentil lectin or immunoaffinity columns according to the protocol described by Gilljam (4). ELISA plates were coated with 400 ng per well (Nunc-Immuno Plate Maxisorp, 96 wells). The secondary antibody was a rabbit anti-monkey IgG peroxidase conjugate (Sigma) used at a 1:40,000 dilution. The titer of anti-HIV-1 gp120 is defined as the highest dilution of serum that produces twice the optical density (OD) value of the negative control at that dilution.

The presence of neutralizing antibodies against HIV-1 in the plasma samples was detected by an antigen release assay. In brief, SHIV-HXB virus containing 40 $TCID_{50}$ units was incubated in triplicate with plasma samples at a 1:2 dilution. Plasma samples from an HIV-1–infected patient (1:12) and an uninfected rhesus monkey served as positive and negative controls, respectively, for neutralizing activity. Following 30 min of incubation at room temperature, the samples were mixed with 50,000 CEM×174 cells. The cells were maintained in RPMI 1640 medium containing 10% FCS for 4 days, with fresh medium being added every 2 days. The SIV p27 antigen released into the culture supernatant was measured by the p27 assay, and the average of the three duplicates was determined. As shown in Fig. 3 below, a culture with no plasma present provided an average of 480 pg/ml p27 antigen, whereas cultures without SHIV gave an average of 18 pg/ml antigen as the background values for the assay.

The presence of SHIV in the plasma of immunized/challenged monkeys was analyzed by competitive RT-PCR. A competitor synthetic RNA was generated as follows. A 711–base pair (bp) DNA fragment from SIVmac239 *gag/pol* genes (SIVMM239 nucleotide sequence 2256-2966) (5) was cloned into the pCRII vector. A 60-bp bacterial phage DNA was inserted into a *Stu* I site in the 711-bp SIV *gag/pol* fragment, generating plasmid pEX1-11. The competitor RNA was synthesized from this modified SIV *gag/pol* sequence by in vitro transcription using Sp6 RNA polymerase. Viral particles were pelleted from 1-ml plasma samples by centrifugation at 14,000 rpm for 1 h, from which viral RNA was extracted using the Micro-scale Total RNA Separator Kit (Clontech). Reverse transcription of the viral RNA was carried out in the presence of 0 or 10^4 molecules of the competitor RNA, 5 μM of random hexamer primer (Pharmacia), RNase in-

hibitor, and M-MLV RT (Gibco BRL) at 37°C for 90 min. The cDNA was subsequently used for PCR amplification using a 5' primer (5'ATGGCCAAATGCCCAGACAGAC, SIVMM239 nucleotide sequence 2578) (5) and a 3' primer (5'AGAGAGAATTGAGGTGCAGCA, SIVMM239 nucleotide sequence 2824) (5). A 247-bp fragment was amplified from the SIV viral cDNA, whereas a larger fragment (310 bp) was amplified from the competitor cDNA. PCR samples were analyzed on a 2.5% agarose gel and confirmed by Southern blot using the SIV *gag*-specific DNA probes (data not shown).

RESULTS

Rhesus macaques (*Macaca mulatta*) are more readily available than cynomologous macaques (*Macaca fascicularis*), and a great deal of data has accrued from the use of rhesus monkeys in human vaccine and drug studies. To develop this animal model, SHIV-HXB virus was prepared from infected rhesus monkey PBMC on a large scale, and its infectivity was then determined by $TCID_{50}$ titration in CEM×174 cells. Two rhesus monkeys were intravenously injected with 10,000 $TCID_{50}$ units. As shown in Table 1, SHIV-HXB replicated efficiently in rhesus monkeys as measured by virus isolation from the PBL of the infected animals but has no apparent effect on CD4 cell counts. These observations agree with previously published data showing that the peripheral blood of cynomologous monkeys was permissive for SHIV replication but that no significant effect on the number of $CD4^+$ T cells of the infected animals was seen (2). This data indicates that rhesus macaques may be a useful animal model for the study of SHIV infection.

The minimum amount of virus required for establishing productive replication in rhesus monkeys was determined by intravenous injection of four animals with 4000, 400, 40, or 4 $TCID_{50}$ units, respectively. Table 2 shows that all the animals became infected and that the level of virus load in the peripheral blood was independent of the size of the virus inoculum. In a second experiment, four rhesus monkeys were intravenously injected with 4, 0.4, 0.04, or 0.004 $TCID_{50}$ units, respectively. Table 2 shows that only the animal inoculated with 4 $TCID_{50}$ became infected. On the basis of this data, an intravenous injection dose containing 24 $TCID_{50}$ should have a 99% probability of infecting a rhesus monkey, whereas an injection of 5.6 $TCID_{50}$ of this virus should have a 90% infection probability (6). All the infected animals in Tables 1 and 2 developed serum antibodies against both HIV-1 gp120 and SIV antigens. Cell-mediated immune responses specific to HIV-1 gp120 were also detected in the infected animals (N. Letvin, unpublished observations). Nonetheless, all the animals appeared healthy despite the established virus infection.

TABLE 1. *Detection of SHIV-HXB in PBMCs of* Macaca mulatta *(Mm, rhesus monkeys) and the $CD4^+$ T-cell count*

Week	Mm421 CD4 count	Mm421 virus isolation	Mm337 CD4 count	Mm337 virus isolation
0	1,860	−	396	−
3	1,277	+	637	+
7	819	+	485	+
10	2,176	+	547	+
15	1,802	−	837	−
19	1,320	+	936	+
22	3,574	−	1,411	−
26	2,892	+	1,137	+
30	2,127	−	1,083	+
35	2,085	+	1,133	−
40	2,264	−	946	−
43	2,911	−	816	−
56	3,542	+	1,357	+

Vaccine development can benefit from the availability of correlates of immunity as elucidated in an animal model. Attenuation of the viremia observed in SHIV-infected rhesus macaques might be one predicted consequence of a protective immune response. Currently, most efforts toward determining the appropriate HIV-1 protective antigen have centered on nonreplicating antigens (7), which quite often require the addition of an adjuvant to potentiate their otherwise poor immunogenicity. The ideal combination of antigen and adjuvant are conformed so that important epitopes are maintained and an appropriate immune response is engendered. We have recently described a water-soluble high molecular weight polyelectrolyte phosphazene species (polydicarboxylatophenoxy phosphazene; PCPP) that has remarkable adjuvant properties (8,9). PCPP has facilitated the induction of very high functional antibody titers against several antigens.

The utility of SHIV in testing the efficacy of HIV-1 vaccine candidates was initiated by immunization and challenge of four rhesus monkeys. The animals were immunized intramuscularly with the following 1-ml formulations: animal Mm91069 received 100 μg recombinant HIV-1 SF2 gp120 produced from Chinese hamster ovary cells with 100 μg polyphosphazene (PCPP) as adjuvant; animal Mm91080 received 100 μg formalin-inactivated HIV-1 LAI virus produced from infected CEM×174 cells; and animals Mm91083 and Mm91084 both received 100 μg inactivated HIV-1 LAI virus plus PCPP.

TABLE 2. *Detection of SHIV-IIXB in PBMC of* Macaca mulatta *infected with different doses*[a]

Week	MML3 4600 $TCID_{50}$	MML28 460 $TCID_{50}$	MML9 46 $TCID_{50}$	MMJO28 4 $TCID_{50}$	MM8A2 4 $TCID_{50}$	MMS-88 0.4 $TCID_{50}$	MM8B7 0.04 $TCID_{50}$	MME363 0.004 $TCID_{50}$
2	460	51	910	100	6	ND	ND	ND
3	4,100	8,300	37,000	1,400	460	ND	ND	ND
4	37,000	2,700	37,000	8,300	910	ND	ND	ND
6	280,000	74,000	670,000	280,000	25,000	ND	ND	ND
8	110,000	110,000	670,000	330,000	8,200	ND	ND	ND

[a] The numbers are the calculated PBMC in the dilution at which 50% of the co-cultures became infected as determined by the assay for SIV p27 antigen. The lower the number of PBMC required to establish infection, the higher the virus load in the blood of the infected animals.

By week 5 after the first immunization, only the two animals that received both whole inactivated virus plus PCPP developed serum antibodies against HIV-1 gp120 as measured by either ELISA or Western analysis (Figs. 1 and 2). The anti-gp120 antibody levels of all four animals had fallen below the level of detection by ELISA by week 43 (Fig. 1), and therefore all the animals received a secondary immunization at this time with 100 μg recombinant HIV-1 HXBc2 gp120 produced by a baculovirus expression system plus PCPP. The choice of antigen for the boost was due merely to the unavailability of HIV-1 SF2 gp120 and whole inactivated HIV-1 LAI. The boost significantly increased the level of serum anti-gp120 antibodies in all the animals. Animals immunized with inactivated HIV-1 virus plus PCPP (Mm91083 and Mm91084) had the highest titers, which were significantly higher than those seen in the other two animals. The antibody response in the vaccinees was confirmed by Western blot analysis using a commercial HIV-1 diagnostic kit. As shown in Fig. 2, the two animals that received inactivated virus plus PCPP developed antibodies against HIV-1 gag proteins that were detectable by week 5 and persisted until week 43. The second immunization merely increased their response to gp120. The adjuvant effect of PCPP on the immunogenicity of HIV-1 antigens appeared to be significant.

A third immunization identical to the formulation as in the second immunization was administrated to all the animals at week 73. Two weeks later (week 75), all the vaccinees were challenged intravenously with 24 $TCID_{50}$ units of SHIV-HBX, which was previously calculated to have a 99% probability of establishing infection. None of the four animals had significantly neutralizing activity in their plasma against SHIV infection at the time of SHIV challenge (Fig. 3). Blood samples were collected from the animals at 3-week intervals after the SHIV challenge and analyzed for SHIV replication by peripheral blood co-culture with human T cells (Table 3). Viruses were isolated from the PBL of animal Mm91069 in the first 2 weeks following SHIV injection, and from the PBL of animal Mm91080 became infected between weeks 2 and 5 after the injection of the SHIV challenge virus. The other two animals, Mm91083 and Mm91084, remained virus isolation negative throughout the experiment (week 24 postchallenge). PCR analysis of the blood samples measuring viral RNA in the plasma (Table 4) confirmed the virus isolation data. Western blot analysis of serum samples from the challenged animals compared sera taken from the animals before and after the SHIV challenge (Fig. 4). The protected animals, Mm91083 and Mm91084, showed no detectable changes in their antibody profile. The unprotected animals, Mm91069 and Mm91080, developed antibodies against HIV-1 gp41 monomer and tetramers, as well as antibodies against the p24 Gag protein (Fig. 4). Because the four animals differed only in the formulation used in the primary immunization, it is important to note that the two protected animals were both immunized with inactivated HIV-1 plus PCPP (Mm91083 and Mm91084). Whether this was due to PCPP as the chosen adjuvant requires further study, as the unprotected Mm91080 lacked any comparable adjuvant in the primary immunization. It is also noted that animal Mm91069 received recombinant gp120 from different strains of HIV-1 in the primary and both boost immunizations, which may have resulted in lack of protection. Nonetheless, by showing evidence of protection this experiment demonstrated that the aforementioned monkey model can be used to test HIV-1 vaccine candidates.

DISCUSSION

In the past 10 years, several HIV-1 vaccine candidates have been tested in chimpanzees. Some

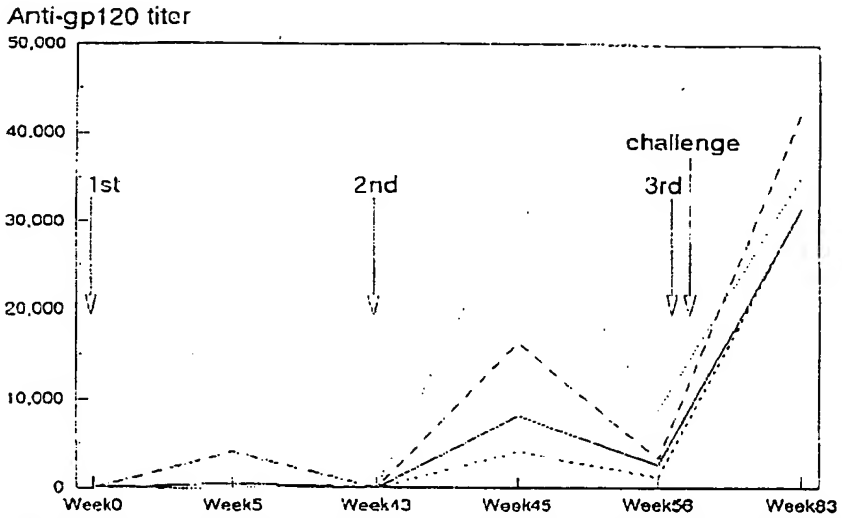

FIG. 1. Serum anti-HIV-1 gp120 antibody ELISA titer of the immunized monkeys. Titer is defined as the highest dilution of serum sample which gives twice the value of the negative control OD at that dilution. Mm91069, 100μg HIV-1 SF2 gp120+PCPP; Mm91080, 100 μg HIV-1 LAI whole inactivated virus; Mm91083 and Mm91084, 100 μg HIV-1 LAI whole inactivated virus + PCPP. All four animals were boosted twice with 100 μg HIV-1 HXBc2 gp120+PCPP and challenged with 24 TCID50 of live SHIV-HXB by intravenous injection. The first immunization occurred at week 0. The second immunization was administered at week 43. The third immunization was given at week 71, and the SHIV-HXB2 challenge was administered at week 73 after the first immunization. Mm91069, solid line; Mm91080, short dashed line; Mm91083, dotted line; Mm91084, long dashed line.

have produced promising results (10–12). From the vaccine development point of view, further studies should test optimal vaccine formulations, including a comparison of different adjuvants, the antigen doses and the optimal schedule of immunization to stimulate maximal protection against challenge by different HIV-1 strains. However, it is almost impossible to conduct these necessary studies in statistically significant numbers of chimpanzees. According to a report from the AIDS Vaccine Surveillance System (13), the total number of chimpanzees used in HIV-1 vaccine challenge studies by 1992

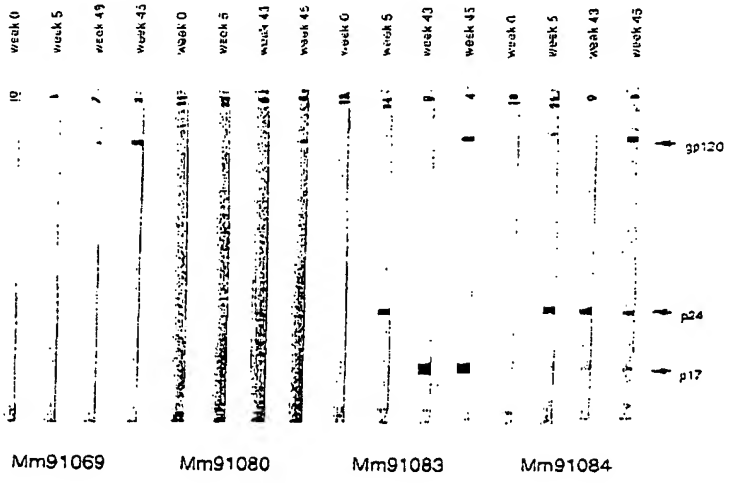

FIG. 2. Western blot analysis of serum samples from the immunized monkeys. Mm91069, 100 μg HIV-1 SF2 gp120+PCPP; Mm91080, 100 μg HIV-1 LAI whole inactivated virus; Mm91083 and Mm91084, 100 μg HIV-1 LAI whole inactivated virus + PCPP. Week 0 was pre-immunization. Week 5 was 5 weeks after the first immunization. Week 43 was the week before the second immunization. Week 45 was 2 weeks after the second immunization.

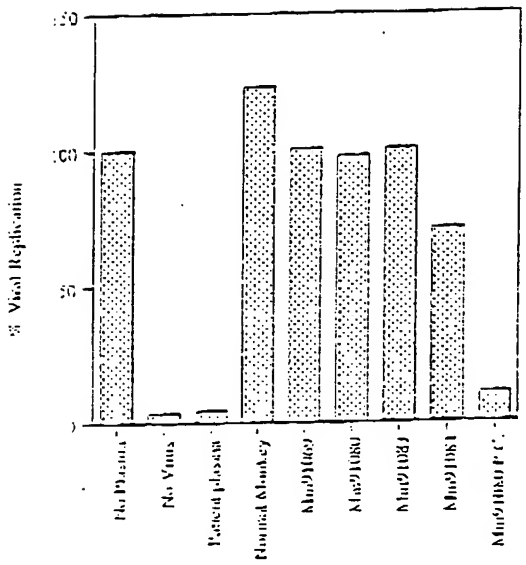

FIG. 3. Neutralization antibody analysis. The level of p27 antigen in the absence of any plasma is defined as 100% viral replication. No virus represents the background p27 level (3.8%). A HIV-1 patient plasma sample decreased the viral replication to 4.7%. A normal monkey plasma, 123.3%. The plasma from vaccinated monkeys was collected at the day before SHIV challenge. Mm91069 (100.5%), Mm91080 (97.7%), Mm91083 (100.6%), and Mm91084 (71.4%). Mm91080 P.C. (10.9%) is the plasma collected 12 weeks after the SHIV challenge.

was 32. These studies include at least five different types of antigens, 14 different protocols, and several adjuvants. It is likely that such variations in inoculum plus the limited number of animals involved in each experiment may contribute to the overall low protection rate (33%) (13). Nonetheless, the data suggests that some vaccine protection may be possible if one can use enough animals to determine optimal formulation.

TABLE 3. *Virus isolation by co-culture of PBMC from immunized and challenged monkeys*[a]

	Weeks postchallenge							
	0	2	5	8	12	16	20	24
Mm91069	—	+	+	+	+	+	—	—
Mm91080	—	—	+	—	+	+	—	—
Mm91083	—	—	—	—	—	—	—	—
Mm91084	—	—	—	—	—	—	—	—

[a] Virus isolation positive is defined as the OD value that is at least twice of that of the background. The serum samples and the negative background were measured in duplicate.

The search for an effective SIV vaccine to protect monkeys from SIV challenge has produced very important guidelines for HIV-1 vaccine development. These studies indicate a cumulative protection rate of 60% (110/179) (13). However, the fact that a human antigen present in both the vaccine formulation and the SIV challenge stocks played an important role in the protection observed in many of these studies requires careful reevaluation of the previous SIV protection data (14). A number of recent studies reported that whole inactivated SIV virus was able to stimulate protective immunity without involvement of the human antigens (15,16). However, the efficacy of inactivated vaccines appears to be very low according to a study published by the European Community Concerted Actions involving a total of 98 macaques (17).

The success of live attenuated SIV as a vaccine in rhesus monkeys suggests an alternative approach for HIV-1 vaccine development (18). A recent report indicates that the same attenuate vaccine strain behaves very differently in an infant host as compared to an adult (19). The evaluation of this approach in humans may be difficult.

The studies presented here demonstrate the potential utility of an SHIV animal model in testing the efficacy of HIV-1 vaccine candidates. Additional SHIV chimeric viruses have been constructed using envelope genes from HIV-1 ELI, HIV-1 MN, and HIV-1 89.6. Each virus stock was propagated in rhesus monkey PBMC and their $TCID_{50}$ determined. Both SHIV-MN and SHIV-89.6 replicate productively in rhesus monkeys, and SHIV-89.6 demonstrates efficient nontraumatic vaginal mucosal penetration and infection (Y. Lu and C. Miller, unpublished observation). A range of SHIV constructs titrated and characterized for growth in macaques permits the evaluation of a breadth of immune protection afforded by different antigen formulations against challenge with different iso-

TABLE 4. *Plasma viremia of challenged monkeys*[a]

	Weeks post SHIV challenge			
	0	5	12	16
Mm91069	—	200,000	800,000	800,000
Mm91080	—	800,000	200,000	200,000
Mm91083	—	—	—	—
Mm91084	—	—	—	—

[a] The numbers are the viral RNA copies in 1 ml of plasma from different times after the live SHIV-HXB challenge. The negative sign (−) means that the viral RNA copies were undetectable under the conditions used.

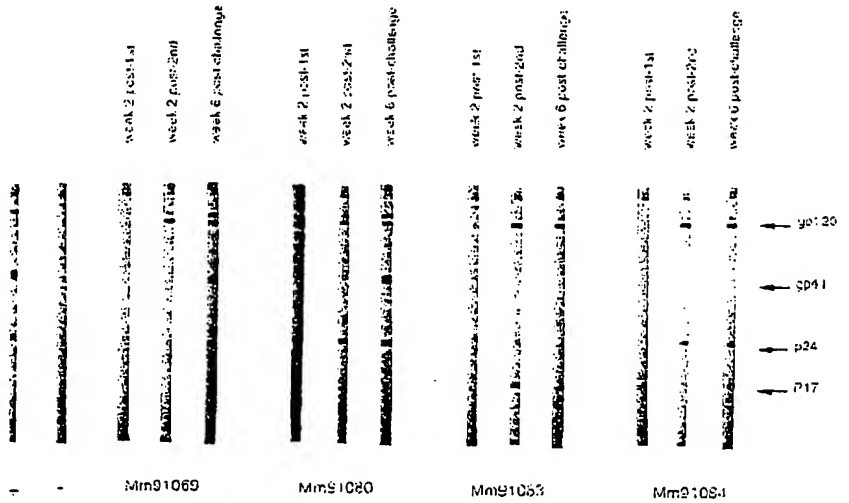

FIG. 4. Western blot analysis of serum samples from immunized monkeys before and after the live SHIV-HXB challenge. −, positive control using SHIV-infected monkeys serum; +, negative control using normal monkey serum. Week 2 post-1st, serum from 2 weeks after the first immunization; week 2 post-2nd, serum from 2 weeks after the second immunization; week 6 post-challenge, serum from 6 weeks after the challenge.

lates and by different routes of infection. Even though this study utilized a limited number of animals, it suggests that it may be possible to stimulate protective immunity in rhesus monkeys against SHIV infection by using inactivated HIV-1 plus a novel adjuvant. If this data is confirmed by a larger animal trial, one can evaluate the substitution of whole inactivated virus with different kinds of virus-like particles, or recombinant antigens that bear structural similarity to their viral counterparts and are safer to use in humans.

It is obvious that the relevance of this animal model to HIV-1 infection in humans needs to be further clarified. Given the absence of clearly defined in vitro correlates of protection against HIV-1 infection and the limited availability of chimpanzees, further exploration of the aforementioned rhesus monkey model for the preclinical evaluation of potential HIV-1 vaccine formulations is warranted.

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Poly[di(carboxylatophenoxy)phosphazene] (PCPP) is a potent immunoadjuvant for an influenza vaccine

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The adjuvant activity of poly[di(carboxylatophenoxy)phosphazene] (PCPP) on the immunogenicity of formalin-inactivated influenza virions and commercial trivalent influenza vaccine was studied. Regardless of which antigen preparation is used, the addition of 100 μg PCPP enhances the HAI antibody response 10-fold over the levels elicited by the vaccine alone. Similarly, PCPP enhanced the IgM, IgG, and IgG1 ELISA antibody titers to influenza antigens at least 10-fold higher than the vaccine alone. In contrast, the IgG2a isotype titers were only enhanced about 2-fold. Immunization of aged mice (22 months old) with trivalent influenza vaccine alone did not sero-convert these mice as measured by HAI or ELISA whereas significant sero-conversion was achieved when mice were immunized with PCPP-formulated trivalent vaccine. The adjuvant activity of PCPP was shown to not be due to a site of injection depot effect. PCPP adjuvanticity was positively correlated to the molecular weight of the polymer. © 1997 Elsevier Science Ltd.

Keywords: adjuvant; polyphosphazene; influenza

Viral vaccines have traditionally been composed of either live attenuated virus or nonreplicating antigens. The latter contain either chemically inactivated virus particles, subviral components or, more recently, recombinantly expressed viral subunits. The nonreplicating vaccines are generally rather weakly immunogenic compared to the replicating attenuated vaccines and, thus, usually require the presence of an adjuvant to enhance immunogenicity. Although relatively weak, the only adjuvant currently used in commercial human vaccines is alum. A notable exception to this is the use of influenza vaccine in an unadjuvanted form.

Trivalent inactivated influenza vaccines are currently recommended[1] for the prevention of influenza in individuals considered at high risk for the development of complications, including death, following influenza virus infection. While these vaccines have reduced the frequency and severity of influenza illness as well as the frequency of hospitalization and death[2], many of those considered at high risk fail to develop adequate immunity following immunization[3]. Unfortunately, alum does not significantly improve the vaccine's efficacy. Consequently, considerable effort has been expended on adjuvant development to improve this vaccine but so far, no safe and effective new adjuvant is available.

A wide range of polyelectrolytes of various molecular weights has been shown to have an adjuvant activity[4]. Macromolecules bearing either positive or negative charges have displayed a similar immunostimulatory activity. The polyelectrolytes form complexes with antigens through electrostatic and hydrophobic bonds. On the other hand, neutral polymers have had no effect on the immune response unless the uncharged polymers were conjugated to the protein antigens. A new class of ion cross-linkable water-soluble polymers, the ionotropic polyphosphazenes, was recently described[5]. Since anionic and cationic polymers have previously been shown to have immunoadjuvant activity, we previously investigated the immunogenicity of influenza antigens in solutions of a poly[di(carboxylatophenoxy)phosphazene], PCPP, in the absence of ionic cross-linking[6]. The present experimental results confirm the potent adjuvanticity of PCPP and extend our knowledge of some parameters of the PCPP-enhanced immune response.

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MATERIALS AND METHODS

Synthesis and purification of PCPP

Poly[di(carboxylatophenoxy)phosphazene] (PCPP) was synthesized by a molecular substitution process as

originally described[5]. The PCPP was dissolved in pH 11 sodium carbonate buffer and purified by preparative column chromatography on a 3–4-l Sephadex G-10 column, pH 7.4. These chromatography conditions gave remarkably good separation of PCPP from the contaminants as determined by HPLC, NMR and elemental analysis. Lyophilization of the PCPP-containing fractions yielded a perfectly white, fluffy, amorphous material that was chromatographically homogeneous by HPLC. Approximately 1.5 g was purified per chromatographic run. The molecular weight (M_w) of the PCPP ranged from approximately 800–1200 kDa as determined by multiangle laser light-scattering (MALLS) detector DAWN DSP-F (Wyatt Technology, Santa Barbara, CA)[7]. PCPP solutions were prepared by dissolving the appropriate amount of PCPP in phosphate-buffered saline (PBS), pH 7.4 at 80°C with agitation. The antigen solution was then slowly added to the polymer solution so that the final concentration of PCPP was 0.1% (100 μg (0.1 ml)$^{-1}$).

Immunogens

Influenza virus strains PR8 (H1N1) (Spafas, Storrs, CT) and X-31 (PR8 reassorted with H3N2) (Spafas) were grown in the allantoic cavities of embryonated chicken eggs according to standard methods. The virus was purified from the allantoic fluid by sucrose gradient ultracentrifugation and quantitated by protein, hemagglutination and plaque assays. Influenza was formalin-inactivated by the addition of a 38% formaldehyde solution at a final dilution of 1:4000 and incubated at 4°C for 24 h. Formalin-treated virus was tested by blind passage two times in the allantoic sac to insure the complete inactivation of the virus before its use as an immunogen. Trivalent Fluzone (A/Texas/36/91 (H1N1)), A/Shangdong/9/93 (H3N2), B/Panama/45/90) (Connaught, Swiftwater, PA) was purified from allantoic fluids by continuous sucrose gradient centrifugation, was formalin-inactivated and was treated with Triton X-100 to produce a 'split antigen'. The antigen was then further purified by chemical means and 0.5-ml aliquots dispensed in vials to contain one human dose of 15 μg HA for each virus strain.

Parenteral immunization

Polymer–antigen formulations were prepared as described above. Female 7–8 week old BALB/c mice were randomized into groups. Mice were immunized subcutaneously with 0.1 ml containing 100 μg PCPP, by means of a 25 gauge needle, in the loose skin over the neck. In those experiments where a booster immunization was administered, the same antigen formulation and immunization procedure was used. Blood samples were taken from the retroorbital sinus of CO_2 anesthetized mice.

Immune response assays

Antigen-specific antibodies in mouse serum were determined by ELISA in 96-well microtiter plates coated with the appropriate antigen in sodium carbonate buffer, pH 9.6. The influenza antigens used in the immunological assays were either virus purified from allantoic fluids or cell extracts from virus-infected Madin and Darby canine kidney cell (MDCK) monolayers using standard cultivation methods. Sites available for nonspecific binding of protein after coating and washing were blocked by adding 2.5% bovine serum albumin (BSA; Sigma, St Louis, MO) in PBS. After blocking and washing, twofold serial dilutions of sera in 1% BSA/PBS were added to the wells. Unbound serum was washed away and horseradish peroxidase-labelled goat anti-mouse IgG (Sigma, St Louis, MO) was added for 1 h. Unbound conjugate was washed away and serum antibody was detected by adding the substrate *o*-phenylenediamine dihydrochloride (Sigma). The reaction was stopped by addition of 2 M H_2SO_4 and the absorbance read at 490 nm. The endpoint titers were the reciprocal of the highest sample dilution producing a signal twofold greater than that of an antibody-negative sample at the same dilution.

The Ig class and IgG isotypes of the ELISA-reactive influenza-specific antibodies were determined by the detection of murine antibodies bound to the antigens. To do this, horseradish peroxidase-labelled sheep anti-mouse antibody specific for mouse IgM, IgG classes and IgG subclasses 1, 2a, (Boehringer Mannheim) was reacted with the mouse antibodies bound to the antigen in the ELISA plates.

The influenza hemagglutination-inhibition antibody assay was done with heat-inactivated mouse serum that had been incubated for 30 min with 10% chicken red blood cells (Crane) to remove nonspecific inhibitors. Twofold dilutions of sera were added to 96-well microtiter plates and 8 HA units of virus suspension in an equal volume were added to each well and incubated at room temperature for 30 min. A 0.5% suspension of chicken red-blood cells was added to each well and incubated at room temperature for 45–60 min. The HAI titers were expressed as the reciprocal of the highest dilution that completely inhibited hemagglutination of erythrocytes.

Statistical analysis

All antibody titers were expressed as geometric mean titers (GMT). Data were statistically analyzed by Student's *t*-test of unpaired samples using a Statistica/Mac software (StatSoft, Inc., Tulsa, OK), with probability values, P, of <0.05 treated as significant. Unless otherwise stated, there were five to eight mice per group in all experiments.

RESULTS

The effect of PCPP on the immunogenicity of influenza virions

The adjuvant activity of PCPP was examined by mixing the aqueous polymer solution with formalin-inactivated X-31 influenza virus particle suspension and injecting 0.1 ml parenterally into mice. This inactivated whole-virus preparation contained approximately 5 μg total virus protein. Groups of mice were subcutaneously immunized with 0.1 ml containing 5 μg formalin-inactivated X-31 influenza virus formulated in either PBS or 100 μg PCPP. The effect of PCPP on the IgG and IgM response was monitored by following the kinetics of the antibody response as measured by ELISA. The antigen employed in the IgG ELISA was

were subcutaneously immunized with one human dose of Fluzone containing 15 μg hemagglutinin from each virus strain (Texas, Shangdong, Panama). The trivalent antigen was administered either without any further formulation or formulated with 100 μg PCPP. The antibody titers elicited by the vaccine and the vaccine formulated with PCPP were monitored at 3, 6 and 15 weeks post-immunization using the HAI (*Figure 3*a) and ELISA (*Figure 3*b,c) assays.

The presence of PCPP in the vaccine formulation dramatically enhanced the immune response to all three influenza HA strains compared to the commercial vaccine formulation (*Figure 3*a). At 6 and 15 weeks post-immunization, the HAI antibody titers induced by the PCPP formulation were approximately 10 times higher than for the commercial vaccine. This increase is in the same order of magnitude as what was observed for the 5-μg dose of formalin-inactivated influenza immunogen (*Figure 1*). The recorded HAI titers were higher for Texas than Shangdong which in turn were higher than the Panama responses for both vaccine formulations. The kinetics of the HAI response indicate that the antibody titers continue to increase between 6 and 15 weeks. An HAI response of almost 5120 to the Texas strain was detected at week 15 in the group of mice immunized with the PCPP adjuvant formulation, whereas the trivalent vaccine alone at this time point elicited a titer of 640. In a mouse potency test, an antigen dose that induces HAI antibody titers ≥40 units is predictive of protection in a human[3].

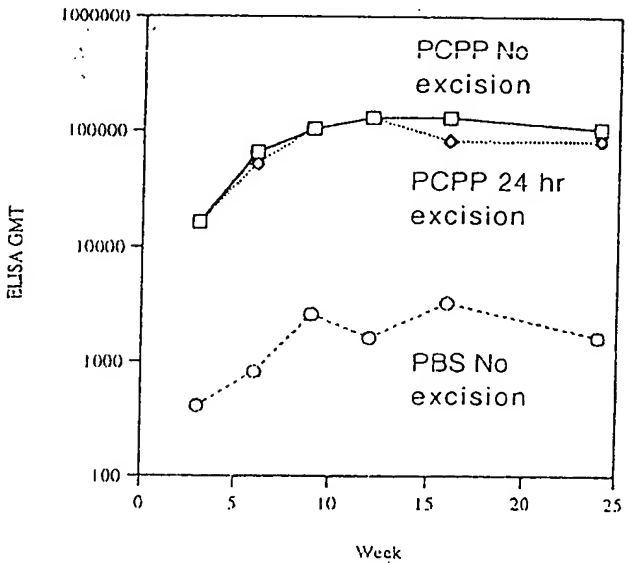

Figure 2 Effect of injection-site excision on the immune response. Groups of three BALB/c mice were subcutaneously immunized with a 0.1-ml volume containing 5 μg purified formalin-inactivated X-31 influenza virus formulated in either PBS or 100 μg PCPP. The site of immunization was marked with a permanent black marker. At 24 h post-immunization, one group of mice that received PCPP-formulated influenza was anesthetized and the entire palpable injection site extirpated and the wound closed with sutures. No surgical intervention was performed on the two remaining groups that had been immunized with either PCPP- or PBS-formulated virus. The influenza-specific ELISA antibodies were monitored periodically over the next 24 weeks. ELISA plates were coated with purified live X-31 influenza. Significance testing at week 16 for PBS no excision and PCPP no excision was $P = 0.003$ and PCPP excision and PCPP no excision was $P = 0.52$ (not significant)

Additional animal immunization experiments have shown that PCPP is equally effective in the induction of ELISA and HAI antibodies when administered by the intramuscular route (data not shown). These results demonstrate that PCPP is a very potent adjuvant with the mouse effective dose equal to 100 μg when used with a human dose of the commercial influenza vaccine.

The IgG antibody isotypes induced by these formulations were determined by an ELISA assay. Both the PBS and PCPP influenza formulations elicited IgG1

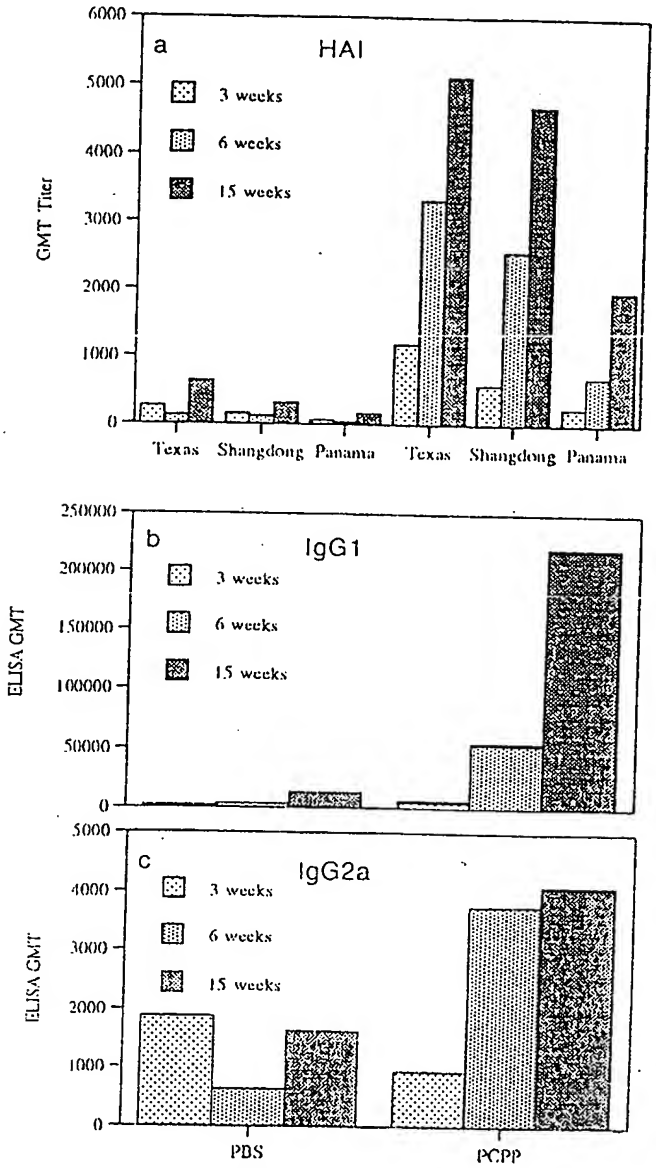

Figure 3 The effect of PCPP on the immune response to trivalent influenza. BALB/c mice (five per group) were subcutaneously immunized with a 0.5-ml volume of trivalent (A/Texas/36/91 (H1N1)), A/Shangdong/9/93 (H3N2), B/Panama/45/90) influenza vaccine alone (0 μg PCPP) or vaccine formulated with 100 μg PCPP. The immunogen dose was 15 μg HA for each of the three vaccine strains. Serum samples were collected at 3, 6 and 15 weeks post-immunization and the influenza-specific HAI titers to each virus subtype (a) and the IgG1 (b) and IgG2a (c) isotypes to influenza/Texas were determined. Significance testing at week 15 for PBS and PCPP formulations measured by HAI titers was Texas $P = 0.0001$, Shangdong $P = 0.0000001$, Panama $P = 0.00012$, and for ELISA titers was IgG1 $P = 0.0000093$ and IgG2a $P = 0.076$ (not significant)

The immunogenicity of antigens as diverse as influenza, tetanus toxoid, HBs, Hib PRP, HSV gD2, and HIV env is dramatically enhanced in the presence of soluble PCPP. Except for the Hib polysaccharide antigen, a single dose of immunogen mixed with PCPP elicited a persistent high titer antibody response that lasted at least 6 months. This is in contrast to recently developed adjuvants such as QS-21, MPL and MF-59 all of which require three administrations to achieve long-lasting high-titer antibody levels. Furthermore, the data[12] clearly showed that PCPP at very low concentrations is as efficient as complete Freund's adjuvant (CFA) in inducing high serum IgG responses to both of the tested antigens (influenza, tetanus toxoid). Perhaps more importantly, PCPP admixed with influenza antigens out-performed CFA by eliciting high-titer functional antibodies measured by hemagglutination inhibition and infectivity neutralization assays. PCPP has, thus, proved to be a versatile and effective adjuvant.

The present report confirms the previously observed[6,12] induction of high functional antibody titers by PCPP-formulated influenza antigens derived from research-laboratory-produced virus and extends these findings to commercial trivalent influenza vaccine. Regardless of which antigen preparation is used, the addition of 100 μg PCPP enhances the HAI response 10-fold over the levels elicited by the vaccine alone. The induction of high functional antibody titers is presumably due to the maintenance of antigenic integrity in the presence of PCPP. Similarly, PCPP enhanced the IgM, IgG, and IgG1 antibody titers to influenza antigens at least 10-fold higher than the commercial vaccine alone. In contrast, the IgG2a isotype titers were only enhanced about 2-fold.

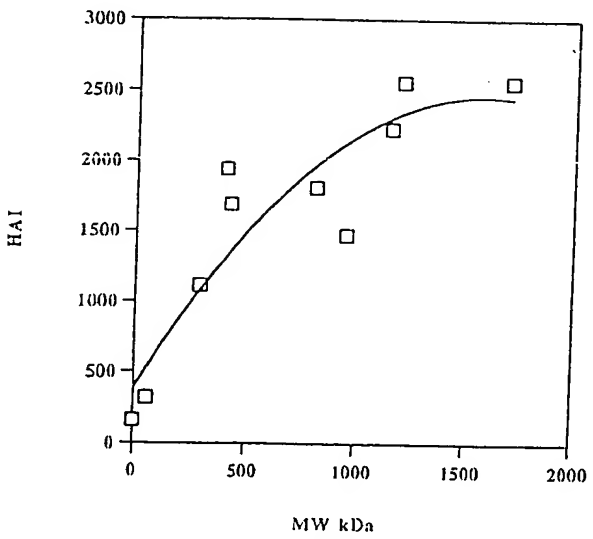

Figure 5 The effect of PCPP molecular weight on the immune response. PCPP preparations having various molecular weights as determined by HPLC analysis were synthesized and purified as described in Materials and Methods. The nine PCPP samples had relatively narrow polydispersities (ranging from 1.3–1.87) and average molecular weights ranging from 58–1700 kDa. Each PCPP material (100 μg) was mixed with trivalent influenza vaccine (A/Texas/36/91 (H1N1)), A/Shangdong/9/93 (H3N2), B/Panama/45/90) and a 0.5-ml volume was injected subcutaneously into mice (five per group). The immunogen dose was 15 μg HA for each of the three vaccine strains. As a control (0 μg PCPP), another group of mice was injected with trivalent influenza vaccine formulated in PBS. The individual HAI antibody responses against all three virus strains were assayed at 9 weeks post-immunization. The HAI data for each virus strain was computer-analyzed and found to fit a second-order polynomial equation. The molecular-weight-dependence of the antibody response is exemplified by the results obtained with influenza strain Texas. Significance testing of a PBS formulation compared to each PCPP molecular weight formulation was the following: 58 kDa $P = 0.105$ (not significant) and all molecular weights from 290–1700 kDa inclusive were at least $P < 0.002$

There is a growing body of evidence showing that antigen persisting on the external membrane of follicular dendritic cells in the lymph nodes is involved in the recruitment of B memory cells to form antibody-secreting cells, induction of high antibody titers, affinity maturation and long-term memory[13]. Acceptance of the antigen persistence concept has an important implication in vaccine development. Ideally, it would be advantageous to be able to formulate vaccines in a way such that antigen is presented to the immune system and in particular the follicular dendritic cells over an extended period of time. Three indirect lines of evidence support a role for PCPP in aiding antigen to persist, albeit at an unknown site. It was previously shown that a single immunization dose with PCPP-formulated antigens induced and maintained very high antibody levels to influenza without the need for booster doses[6]. Furthermore, a 0.04-μg influenza dose in PCPP induced the first detectable antibody levels at week 25 compared to week 3 for a 1-μg influenza dose in PCPP. In the present report, a single influenza antigen dose formulated in PCPP, in contrast to PBS-formulated influenza, induced a long-lived IgM response. Taken together, the data can be interpreted as evidence for the persistence of antigen.

For unknown reasons, immunocompetence deteriorates with age, causing increased morbidity and mortality to infections in the aged. Annual influenza immunization is highly effective in young healthy adults but fails to generate protective immunity in 30–50% of older persons[3,14]. An old-mouse model has been used to show that an age-related decline in the immune response can be at least partially compensated by immunizing with a recombinant vaccinia virus expressing the influenza hemagglutinin[15] or co-administering dehydroepiandrosterone (DHEA) with the influenza vaccine[16]. The present experiments demonstrate that the addition of PCPP to the commercial trivalent influenza vaccine augments the immune response to such an extent that non-responder mice become responders both in terms of the number of mice that sero-convert and in the antibody titer that is achieved. The cause of the PCPP-related reversal of immunosenescence is not known. The mechanism may be related to an enhanced antigen persistence and presentation to immunocompetent cells.

Most adjuvants fail to reach the market place because of reactogenicity particularly at the site of injection. Our experiments have shown that PCPP moves out of the site of injection within 24 h of injection. This may be due to the water-soluble nature of PCPP. Regardless of the mechanism of its rapid exit from the injection site, this characteristic is a distinct advantage in reducing the reactogenicity at the site of injection. The combined properties of high adjuvanticity and negligible reactogenicity make PCPP a

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Review

Bartonella henselae, B. quintana, and *B. bacilliformis*: historical pathogens of emerging significance

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ABSTRACT – *Bartonella* species were virtually unrecognized as modern pathogens of humans until the last decade. However, identification of *Bartonella* species as the agents of cat-scratch disease, bacillary angiomatosis, urban trench fever, and possible novel presentations of Carrion's disease has left little doubt of the emerging medical importance of this genus of organisms. The three primary human pathogenic bartonellae, *Bartonella bacilliformis* (Carrion's disease), *B. henselae* (cat-scratch disease), and *B. quintana* (trench fever), present noteworthy comparisons in the epidemiology, natural history, pathology, and host-microbe interaction that this review will briefly explore. © 2000 Éditions scientifiques et médicales Elsevier SAS

Bartonella / pathology / immunity / pathogenesis / epidemiology

1. Natural history

For many years, the genus *Bartonella* was formally recognized as a single species: *Bartonella bacilliformis*, the causative agent of Carrion's disease, also known as bartonellosis, Oroya fever, and verruga peruana. However, recent genotypic analysis has clearly shown that *B. quintana* (formerly recognized as *Rickettsia quintana* or more recently *Rochalimaea quintana*) and *B. henselae* are also members of the genus *Bartonella* [1, 2]. Although these three *Bartonella* species remain the primary sources of recognized *Bartonella*-associated human disease, a wide variety of *Bartonella* species and genotypes have recently been described from nonhuman vertebrates, and at least some of these can be the source of zoonotic infections. For example, *B. elizabethae* has been isolated from a patient with endocarditis and has been shown to have reservoirs in Old World rats of the genus *Rattus* [3, 4]. *B. clarridgeiae* has been isolated from cats associated with human cases of cat-scratch disease [5–7]; however, in one such example, *B. henselae* was the isolate made from the human patient [5]. PCR-amplified DNA evidence exists for *B. grahamii*-associated human neuroretinitis [8], and serologic evidence has been presented for *B. elizabethae* involvement with human neuroretinitis [9]. Confirmation of *Bartonella* species by isolation from the infected patient remains the gold standard for helping fulfill Koch's postulate with regard to *Bartonella*-associated human disease. However, it is not surprising that isolation of bartonellae from human specimens, which in the case of cat-scratch disease remained elusive for at least 50 years, is still not considered a routine event in many clinical laboratories. Although this review will primarily focus on *B. bacilliformis, B. quintana,* and *B. henselae,* it is likely that additional examples of *Bartonella*-associated diseases will become more commonly recognized in the future.

1.1. B. bacilliformis

As mentioned previously, *B. bacilliformis* was the first infectious agent of the genus *Bartonella* to be formally described as a species. During the late 19th and early 20th centuries, the diseases caused by *B. bacilliformis* were major impediments to the European settlement of western South America. In fact, there is credible speculation that bartonellosis was well recognized among pre-Columbian Native Americans [10]. Perhaps the most dramatic recorded outbreak of human disease thought to be caused by *B. bacilliformis* occurred among laborers involved with the construction of railway lines through the foothills of the Andes mountains of Peru in 1871, during which it is believed that thousands perished [11]. Epidemiologic and natural history issues associated with the disease were clearly of considerable interest to the pioneers of infectious disease theory. The classic form of the disease is biphasic and consists of a life-threatening febrile anemic

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phase which, if the patient survives, may be followed by a secondary episode, characterized by vasoproliferative eruptions known as verruga [12–14]. The common infectious association between the two disease phases was dramatically fused when a Peruvian medical student, Daniel Carrión, inoculated himself with material from a verruga skin lesion (presumably with the belief that the two disease phases were unrelated) and subsequently died of febrile anemia. Remarkably, the human experiment was repeated under the direction of a group of doubtful investigators during a Harvard University expedition to Peru in 1913; however, this time the single 'volunteer' did not die, leading the responsible researchers to seriously (and incorrectly) question the etiologic link between the disease syndromes [14].

No convincing evidence exists to implicate a nonprimate animal reservoir for Carrion's disease and to date no nonhuman vertebrate reservoir for *B. bacilliformis* has been recognized. A large body of early medical entomologic analysis suggests that *B. bacilliformis* is transmitted to humans via the bite of a sand fly of the genus *Lutzomyia* (various *Lutzomyia* spp. are also vectors for leishmanisis over wide regions of the Old and New Worlds). It had been suspected that the limited geographic distribution of a specific sandfly species, *Lutzomyia verrucarum*, was responsible for the apparent limited distribution of the human disease within a certain altitude stratum of the Andean foothills referred to as the 'verruga zone.'

However, recent events have demonstrated that Carrion's disease can occur outside the recognized verruga zone, in areas where *L. verrucarum* is not the dominant sand fly species [15]. In addition, asymptomatic but culture-positive individuals have been identified in proximity to patients with active disease [15]. Moreover, there is an absence of the verruga lesion-producing stage in recent human disease episodes outside the verruga zone. These observations raise troubling questions. For example, can humans with asymptomatic bacteremia serve as adequate reservoirs for disease transmission to other humans in the possible absence of a nonhuman vertebrate reservoir? Do these new isolates represent possible emergent strains of *B. bacilliformis*, which may be able to exploit additional vector species and thus vastly increase the distribution of human disease perhaps with an altered clinical presentation?

1.2. B. quintana

B. quintana was a leading cause of infectious morbidity among Allied troops on the Western front during World War I, along with the agents of epidemic typhus and pandemic influenza. Trench fever is characterized by recurrent, cycling fever and is transmitted among humans by the human body louse, *Pediculus humanus corporis* (as is epidemic typhus). During the World War I era it was demonstrated that body lice could transmit trench fever from persons originally infected many months previously. This apparent close association with long-term human transmissibility and the absence of any as-yet-recognized nonhuman vertebrate reservoir suggest that *B. quintana*, perhaps like at least some examples of *B. bacilliformis*, can exist without an intervening nonhuman vertebrate reservoir. The close association with a widely distributed arthropod vector, the body louse, is in contrast to the apparently limited distribution of sand flies thought to be the vectors of *B. bacilliformis*. Under conditions in which personal hygiene is difficult to achieve, trench fever can clearly attain epidemic proportions.

Although almost forgotten to medical science since the end of World War II, *B. quintana* reemerged as an agent of disease toward the end of the 20th century. Foci of trench fever have been identified often among homeless alcoholic men in Europe and North America [16, 17] and have been referred to as 'urban trench fever' [17]. Perhaps as expected, lousiness is a significant risk factor for *B. quintana*-associated bacillary angiomatosis and urban trench fever [18]. *B. quintana* has also been shown to be a modern-era agent of disease among the immune-impaired, in whom the disease can be manifest as bacillary angiomatosis, a syndrome characterized by vasoproliferative lesions and which may also be induced by *B. henselae* [19] (see below). *B. henselae*-and *B. quintana*-associated bacillary angiomatosis can be remarkably similar in gross presentation to that observed for presumably otherwise healthy individuals with the verruga stage of *B. bacilliformis*-associated disease (see below).

1.3. B. henselae

Cat-scratch disease presents several contrasts to Carrion's disease and trench fever. As a syndrome, cat-scratch disease has been recognized in at least one of its forms for over 100 years. Cat-scratch disease typically manifests in otherwise healthy persons as prolonged regional lymphadenopathy, although more serious complications are well documented and may occur with greater frequency than previously suspected. There has long been a clear association between cat-scratch disease and traumatic wounds associated with the scratch of a cat. Presumably, pioneering clinicians familiar with either trench fever or Carrion's disease were likewise aware of the classic forms of cat-scratch disease. However, unlike either Carrion's disease or trench fever, the hunt for the etiologic agent of cat-scratch disease remained one of the classic unsolved mysteries of microbiology until only recently [20, 21]. It is perhaps ironic that the causative organism was not first isolated and characterized from what was recognized as a cat-scratch disease patient [22]. Instead, *B. henselae* was first isolated from bacteremic, immune-impaired persons, some of whom presented with recurrent fever [22, 23]. Based on the antigen derived from one of these isolates, serologic testing to assess the risk for *B. henselae* infections among immune-impaired persons led to the first experimental data linking *B. henselae* to cat-scratch disease [24]. Unlike immune-impaired patients with bactermemia, recurrent fever, or bacillary angiomatosis, otherwise healthy cat-scratch disease patients do not appear to have an appreciable bacteremic phase. To date, most isolations of *B. henselae* and most evidence of *B. henselae* DNA from the otherwise healthy cat-scratch disease patients have come from lymph node tissue [25, 26].

B. henselae is a common infection of domestic and feral cats (*Felis cattus*) [27–29] and produces long-term bacte-

Table I. Selected pathologic characteristics of *Bartonella*-associated infections.

Bartonella *species*	*Vasoproliferative lesions*		*Cat scratch lymphadenopathy*	*Hepatosplenic granulomas*	*Endocarditis*	*Bacteremia*
	Cutaneous	*Systemic*				
B. henselae	+	+	+	+	+	+
B. quintana	+	+	-	-	+	+
B. bacilliformis	+	-	-	-	-	+

remia in this species [30]. The distribution of feline infections with *B. henselae* and the cat flea (*Ctenocephalides felis*) appears to be concordant [27], and experimental evidence has demonstrated that cat fleas can transmit *B. henselae* between cats [31]. The potential role of the cat flea as a vector of cat-scratch disease from cats to humans has not been experimentally evaluated.

Transmission of *B. henselae* to humans is obligatorily associated with feline reservoirs. There are an estimated 60 million pet cats in the United States alone. In many geographic areas, 50% of cats have evidence of current or past *B. henselae* infection [27, 28, 32]. The feline, nonhuman reservoir for cat-scratch disease is ubiquitous and often in intimate contact with an enormous pool of potential susceptible humans. An estimated 24 000 cases of cat-scratch disease occur each year in the United States [33]. Cat-scratch disease has all the hallmarks of a classic zoonotic disease with an obligate alternate vertebrate reservoir. Although cat-scratch disease may be among the most common of zoonotic diseases, the requirement for a feline reservoir and close cat contact probably precludes *B. henselae* from achieving the epidemic potential of infectious agents that do not have an obligate vertebrate reservoir requirement (e.g., *B. quintana* and *B. bacilliformis*).

1.4. Emergent *Bartonella* species and zoonotic potential

Large numbers of *Bartonella* species have recently been recognized to exist either primarily or exclusively as nonhuman enzootic disease [34]. This observation, together with the recognition of occasional examples of sporadic zoonotic disease [15], can be used as suggestive evidence that human-associated *Bartonella* diseases have probably emerged as 'accidental' zoonotic infections. It appears as though *B. quintana*-associated disease has made a significant transition from what was once probably an obligate zoonosis, complete with nonhuman alternate reservoir to a disease characterized by long-term, human-to-human transmissibility mediated by a common human-specific arthropod vector, the body louse. Accompanying this hypothetical transition, trench fever appears to have acquired the potential for epidemic transmission among humans under conditions that favor the body louse.

It is interesting to contemplate whether we are currently witnesses to a similar transition for *B. bacilliformis* from an obligate zoonotic cycle to a potentially human epidemic cycle involving long-term human bacteremia with multiple sand fly species capable of transmitting disease from human to human. Likewise, we may be witness to the emergence of human disease typically associated with enzootic bartonellae; for example, the occasional human disease caused by *B. elizabethae* that is associated with what appears to be a well-established component of rat infectious fauna.

2. Pathology of *Bartonella*-associated infections

The spectrum of pathology attributable to *Bartonella*-associated infections is remarkably broad, ranging from vasoproliferative lesions to granulomatous inflammation. Some species of *Bartonella* produce consistently distinct pathology, while others may cause different histopathologic lesions, depending on the immunologic status of the host, the tissue involved, or the temporal evaluation of the lesion. Although abundant data are available on the descriptive pathology of *Bartonella*-associated infections, the pathophysiology of most of these diseases remains poorly understood. *Table I* lists the major pathologic manifestations of *B. henselae, B. quintana,* and *B. bacilliformis* infections.

2.1. B. henselae

Of all the bartonellae, *B. henselae* produces the most diverse recognized assemblage of histopathologic lesions. Although the bacterium that was first isolated and characterized was initially identified as an agent responsible for recurrent fever [22], the same organism was soon associated with bacillary angiomatosis and bacillary peliosis [35, 36]. Within the next few years, *B. henselae* was definitively associated with varied pathologies of cat-scratch disease, including adenopathy [37], hepatosplenic granulomas [38, 39], neuroretinitis [40, 41], encephalopathy [42], oculoglandular cat-scratch disease [43, 44], and osteomyelitis [45, 46]. *B. henselae* has also been identified as an agent of endocarditis [47, 48] and relapsing bacteremia [22, 49].

Bacillary angiomatosis has been observed in a wide range of tissues. The potentially systemic nature of the disease is reflected by involvement of brain, bone, lymph node, bone marrow, skeletal muscle, conjunctiva, and mucosal surfaces of the gastrointestinal and respiratory tracts [17, 19, 50]. Skin lesions are most frequently noted and best described. However, involvement of deep tissues may occur in the absence of cutaneous bacillary angiomatosis [51]. Cutaneous bacillary angiomatosis may occur singly or as multiple lesions; the number of lesions is generally proportional to the degree of immune compromise of the host [50]. Macroscopically, skin lesions appear as pink to deep red or purple papules or nodules [52, 53]. The microscopic appearance of bacillary angiomatosis in

most tissues is characterized by a lobular proliferation of small, rounded vessels lined by plump endothelial cells with variable atypia. The lumens of many of the vessels may not be visible. The vascular spaces are separated by a mucinous or fibrotic stroma, which contains clusters of intact and fragmented neutrophils. One of the defining characteristics of the lesion are the numerous aggregates of bacteria distributed extracellularly within the stroma, typically associated with the neutrophilic infiltrates [50, 52, 53]. By hematoxylin and eosin staining, the bacterial colonies appear as granular purple clusters. However, the organisms are best demonstrated by using silver staining techniques, such as Warthin-Starry, Steiner, or Dieterle methods [50]. Although neutrophils are generally the predominant inflammatory cell type, these cells may be sparse in bacillary angiomatosis lesions in tissues such as lymph nodes [54] or conjunctiva [55]. In the liver and spleen, bacillary angiomatosis appears as multiple dilated and blood-filled spaces lined by flattened or cuboidal endothelium. The large spaces formed in these tissues give rise to the name bacillary peliosis. The peliotic spaces are separated by a fibromyxoid stroma containing clusters of bacteria, but comparatively few neutrophils [36, 56].

Cat-scratch disease, specifically cat-scratch disease adenopathy, was first formally described 52 years before the bacterial etiology was identified [57], and more than 60 years before definitive association with *B. henselae* [24, 37]. The histopathology of cat-scratch disease in lymph nodes is dependent largely on the temporal evaluation of the biopsy. Follicular hyperplasia, focal cortical necrosis, and microabscess formation near the subcapsular sinus characterize early cat-scratch lymphadenitis. Subsequently, the lesions progress to small cortical granulomas as macrophages ring the areas of neutrophilic infiltrate and necrosis. In later lesions, microabscesses within the granulomas coalesce to form the characteristic 'stellate microabscesses' of cat-scratch lymphadenopathy [58]. Eventually, the suppurative component resolves, leaving a central area of necrosis. Bacteria are often identified in early lesions associated with the walls of swollen capillaries in the cortex of the lymph node. Bacteria are most easily visualized in the first two to three weeks after onset of adenopathy and become less numerous as the suppurative component of the lesions progresses [59]. Similar granulomas with stellate microabscess formation may be observed in other sites in patients with cat-scratch disease, including the liver [60, 61], spleen [62], and bone [63].

Parinaud's oculoglandular syndrome is an intriguing complication of classic cat-scratch disease that in some ways may demonstrate a bridge between the vasoproliferative and granulomatous lesions attributable to *B. henselae*. This condition occurs when *B. henselae* bacilli are inoculated onto the conjunctiva of the patient, resulting in conjunctivitis and local adenopathy. Although draining lymph nodes demonstrate characteristic necrotizing stellate granulomas, the conjunctivae show varied degrees of necrosis, vasculitis, granulomatous inflammation, and interestingly, proliferation and dilatation of blood vessels. In these lesions, bacilli are abundantly distributed around blood vessels [59]. This pathology was among the earliest clues that the same agent could be the cause of both bacillary angiomatosis and cat-scratch disease [64].

Reconciling the diverse histopathologic presentations attributable to infection with *B. henselae* has been most easily achieved by evaluating the immune status of the patient. The florid vasoproliferative lesions of bacillary angiomatosis and bacillary peliosis are characteristically manifestations of advanced human immunodeficiency virus type 1 (HIV-) disease, typically occurring in patients with CD4 lymphocyte counts of less than 100 cells/mm^3. Bacillary angiomatosis has also been described in immunosuppressed transplant recipients and in patients receiving cancer chemotherapy [17, 53, 65]. Conversely, granulomatous lesions associated with cat-scratch disease are characteristically observed in persons with otherwise intact immune systems. It has been proposed that a specific *Bartonella* factor may be activated or expressed in persons with defective cellular immunity that elicits endothelial cell proliferation [66]. Indeed, *B. henselae* and *B. quintana* are known to enhance proliferation and migration of vascular endothelium in cell culture, suggesting production of angiogenic factors by these bacteria [67]. This assumption seems plausible, considering that a wide variety of bacterial pathogens, including *Francisella tularensis*, *Chlamydia trachomatis*, *Brucella* spp., *Yersinia enterocolitica* actinomyces, and *Treponema pallidum*, may produce lymph node pathology similar to that of cat-scratch disease [61]. However, none of these organisms are known to produce angiomatous lesions in immunocompromised hosts. Bacillary angiomatosis has also been described in a small number of immunocompetent patients [68–70], suggesting that additional and as-yet-unidentified factors influence the clinical presentation and pathology caused by *B. henselae*.

2.2. B. quintana

B. quintana causes a broad repertoire of diseases, including classic trench fever, chronic bacteremia, bacillary angiomatosis, endocarditis, and chronic lymphadenopathy. Epidemics of trench fever, also known as five-day fever for the periodic nature of fever relapses, occurred during the first and second World Wars. The disease is characterized by fever, severe headache, and pain in the long bones of the legs. Variation in the severity and duration of trench fever has been observed among volunteers experimentally infected with the same inoculum [71], suggesting that differences in host response influence the disease manifestations of *B. quintana*. However, the emergence of *B. quintana* as an agent of endocarditis [17, 72, 73] and bacillary angiomatosis [19] is particularly enigmatic, as neither of these manifestations were described from the more than 1 000 000 persons estimated to have been infected with this pathogen in the first decades of the 20th century [74]. A number of factors related to the bacterium, its vectors, and the human hosts have been considered to account for these apparently novel manifestations. These include genetic polymorphisms that alter virulence in the bacteria, coinfection with other microorganisms that result in vasoproliferative disease, emergence of other arthropod vectors that modify the pathogenesis of

B. quintana infection, and host factors, including human leukocyte antigen haplotype or immune status [75].

B. quintana causes bacillary angiomatosis in immunocompromised hosts, and the histopathologic lesions are indistinguishable from the vasoproliferative lesions induced by *B. henselae*. However, the species of bartonella may influence distribution of these lesions in specific patient tissues; subcutaneous and bone involvement appears strongly associated with *B. quintana*, whereas lymph node involvement and hepatosplenic lesions are almost exclusively associated with *B. henselae* [18]. The reasons for the apparent tissue tropism are unknown. Although *B. quintana* has not been associated with classic cat-scratch disease, this is probably most reflective of its natural history and host range rather than specific histopathologic responses it elicits in its human host. Still, descriptions of granulomatous inflammatory response to this pathogen are relatively uncommon. Granulomatous mediastinal lymphadenopathy has been described in two patients [76, 77]. Similarly, granulomas with central stellate necrosis have been observed in thalamic lesions of patients with central nervous system (CNS) disease [78]. In these patients, infection with *B. quintana* was confirmed by isolation or by detection of specific nucleic acid sequences by using a PCR assay. However, antibody reactive with the organism could not be detected in the serum of any of these patients, supporting the concept that perturbations in the immune response of the host may influence the particular histopathologic response to infection with bartonellae.

Patients infected with *B. quintana* may also develop protracted bacteremia. Early reports of trench fever noted that approximately 5 to 10% of patients with this disease remained symptomatic for months to years after the initial illness [79]. In naturally and experimentally infected patients, *B. quintana* has been isolated several weeks to months after the onset of disease [71, 80] and in one description, the organism was recovered from the blood of a patient eight years after the original infection [81]. Patients with prolonged bacteremia frequently demonstrate delayed or blunted antibody responses to *B. quintana* [71, 80].

2.3. B. bacilliformis

Similarly to *B. henselae* and *B. quintana*, *B. bacilliformis* elaborates an angiogenic factor in vitro [82, 83]. The tissue lesions of verruga peruana are remarkably similar to those of bacillary angiomatosis. As in bacillary angiomatosis, lesions are characterized by lobular proliferations of plump, atypical endothelial cells forming both relatively solid sheets as well as small, well-formed vessels with patent lumens. Inclusions may be found in the cytoplasm of some of the endothelial cells (Rocha-Lima inclusions), which represent phagosomes packed with degenerating bacteria [84]. Clumps of extracellular bacteria may be visualized in the interstitium, often opposite blood vessels. However, the number of organisms is relatively small and does not approach the quantity of bacteria observed in bacillary angiomatosis. Lymphocytes and plasma cells rather than neutrophils are the predominant inflammatory cell types seen in this disease. Verruga peruana is also distinct from bacillary angiomatosis in that the vasoproliferative lesions are confined to the skin and do not involve internal organs [85].

3. Immunology

Despite the lengthy history of *B. bacilliformis*- and *B. quintana*-associated disease, little information on specific immunity to these agents is available. Highlighting this deficiency in immunological information of the genus *Bartonella* has been the inability to culture *B. henselae* until the last decade. Bacillary angiomatosis in immunocompromised patients induced by *B. henselae* and *B. quintana* suggests a role for host immunity in the control of opportunistic disease. However, without analysis and modeling of immune induction, a complete picture of the host-pathogen interaction, as well as protective immunity, remains elusive. In particular, a knowledge gap exists in the relationship between *B. bacilliformis*-, *B. quintana*- and *B. henselae*-specific immune induction. A summary of known immunological factors reactive to bartonellae infections is listed in *table II* and discussed below.

3.1. *B. bacilliformis* and *B. quintana*

Regarding *B. bacilliformis*-induced human immunity, it is believed that long-term protective immunity may be conferred by antibody, and high seroprevalence rates in disease-endemic areas are observed [86]. Indeed, natives of disease-endemic regions appear to be less susceptible to infection and hemolytic disease than persons from outside these areas [14]. Neutralizing antibodies may confer partial or full protective immunity. This is supported by in vitro studies, which show that many outer membrane proteins of *B. bacilliformis* are recognized by immune serum [87], and that antiflagellin antibodies reduce in vitro invasion of erythrocytes by nearly 100% [88]. However, the presence of chronic asymptomatic carriers of this bacterium [15, 89] where the disease is endemic casts doubt on the hypothesis of humoral mediated protective immunity [90]. In addition, a correlation between the development of humoral responses and development of the verruga stage of disease is apparent [91]. The disappearance of the acute febrile stage coincides with the development of antibody titers and the appearance of verruga lesions. In the presence of humoral immunity, the bacteria may retreat to the vascular epithelial tissues after the anemic fever stage of disease, where they reside and later cause the verruga stage of disease [91]. Cellular immunity in the form of cytotoxic T lymphocytes may be required to fully clear the infection from these sequestered sites. Isolation from the blood of possibly chronic carriers [15, 89] supports the theory that bacteria escape the vascular endothelium because of pressure imposed by cellular immune activity [91].

B. quintana was first cultured axenically in the early 1960s and has been underutilized as a tool for development and characterization of immune and infection models of bartonellae. Perhaps most limiting is the inability to infect or isolate this bacterium from any species other than humans. Thus, animal models of *B. quintana* infection

Table II. Immunological characteristics of *Bartonella* infection.

Bartonella *species*	*Host*	*Immunity*	
		Humoral	*Cellular*
B. henselae	Human	+	Granuloma Skin test Lymphadenopathy
	Cat	+	Granuloma Skin test
	Mouse	+	Delayed-type hypersensitivity Cellular proliferation γ-interferon expressionLiver granuloma
B. quintana	Human	+	Granuloma Systemic lesions
	Cat	+	ND*
B. bacilliformis	Human	+	ND*
	Maquaces	+	ND*

* Not determined

have not materialized, and aspects of human immune responses to this agent are ill-defined.

3.2. Bacillary angiomatosis and cat-scratch disease

The identification of *B. henselae* as the agent of cat-scratch disease and bacillary angiomatosis and the reemergence of *B. qunitana* as a second agent of bacillary angiomatosis and urban trench fever have led to an explosion in *Bartonella* species research. Historically, identification of human immune responses to cat-scratch disease was limited to regional lymphadenopathy in proximity to the inoculation papule (cat scratch or bite). In addition, skin testing was frequently used to indicate cellular immunity and thus infection by this agent [92]. In recent years, growth on solid media and cocultivation with eukaryotic cells have facilitated serologic diagnosis. A widely employed indirect immunofluorescence assay was developed, which has provided a routine, laboratory diagnostic test for confirmation of *Bartonella*-associated human disease [24, 93]. However, beyond serology, few studies have addressed detailed aspects of immunity in humans or other host species to *Bartonella* spp. infection.

B. henselae is recognized as causing different immune/disease states during cat-scratch disease and bacillary angiomatosis as described earlier. Despite differences, both cat-scratch disease and bacillary angiomatosis induce the development of systemic antibody against bartonella. In addition, the development of atypical systemic disease caused by *B. henselae* infection has been correlated to immune status of the host [18]. Of particular interest is the development of angiogenic cutaneous lesions in patients with bacillary angiomatois, which are similar to those lesions seen during *B. bacilliformis* infection. This feature may represent deficient immune function in patients with bacillary angiomatosis, since angiogenic lesions are absent in typical cat-scratch disease and trench fever. Unlike patients with the verruga stage of *B. bacillifomis* infection, patients with bacillary angiomatosis may have systemic lesions. Decreased $CD4^+$ T cells as well as decreased phagocytic functions are hallmarks of immunodeficiency caused by HIV. Compromise of these functions may be associated with a variety of opportunistic infections. Impaired phagocytic function could be responsible for the absence of granulocytic formation seen in patients with bacillary angiomatosis, since monocytic cells are associated with this histological response. In cat-scratch disease, the presence of intact immunity may prevent the development of bacillary angiomatosis by containing the organism to the lymph nodes and preventing migration to cutaneous tissues. Studies of host immunity are lacking regarding Carrion's disease. The common development of bacteremia and cutaneous angiogenic lesions may reflect specific virulence capabilities of this organism rather than host immunity alone. However, immunosuppression is often associated with the hemolytic phase of Oroya fever. The reason for this immunosuppression is not known, but the occurrence of secondary infections, such as salmonellosis, shigellosis, toxoplasmosis, tuberculosis, or malaria in Oroya fever cases, suggests immune deficiency [94, 95].

The absence of a well-developed animal model for *B. bacilliformis* or *B. quintana* infection or immunity presents an enormous barrier in studies of immunity to infection with these agents. Until recently, the only animal infection model for *B. henselae* has been the domestic cat, a model for which immunologic reagents are severely limited. In humans, a lack of accessible *Bartonella* antigen reagents limits detailed studies of antigen-specific response including in vitro cellular analysis. And while there are a few reports on cellular responses in humans, most lack significant numbers of subjects or suitable controls. A recent report presented provides evidence that gamma delta T cells may be involved in protection against cat-scratch disease as they are with other intracellular pathogens [96]. A modest increase in gamma delta T cells was observed in two of five patients with cat-scratch disease. More recently, Arvand et al. [97] described specific cellular immunity in a cat-scratch disease patient. Cellular proliferation specific for *B. henselae* was detected by in vitro peripheral

blood cell stimulation. Despite the importance of this detection, the use of only one subject weakens the strength of the observation. However, the authors suggest that the in vitro proliferation assay may be a viable addition to cat-scratch disease diagnosis. Further cellular analysis will surely assist in confirming the role of cellular immunity in cat-scratch disease. However, statistically significant numbers of cases must be studied.

In the absence of additional cellular studies, perhaps the most reliable data on human immune mechanisms involve humoral assays that indicate *Bartonella*-specific cellular immune functions. In this regard, in vitro analysis of complement-mediated lysis of *B. henselae* suggests a lack of enhancement of *Bartonella* killing by specific versus non-*Bartonella* specific antibody [98]. Our own data analyzing human humoral immunoglobulin G (IgG) subtype responses induced during cat-scratch disease suggest little involvement of complement-mediated immunity but support a role for opsonization and subsequent clearance by professional phagocytic cells [99]. In addition, *Bartonella*-specific proteins have been identified that appear to be consistently immunoreactive with serum specimens from cat-scratch disease patients [99, 100]. Whether these proteins represent antigens important to protective immunity remains to be determined. However, the recognition of proteins that react with sera from a large number of cat-scratch disease patients suggests the conservation of at least some antigenic presentation during infection.

The domestic cat has provided an opportunity to study the pathology and immune response to *B. henselae* infection. Reports of naturally infected as well as experimentally infected cats provide a foundation from which to build. High antibody titers are common in *B. henselae*-infected cats, providing an immunological marker for exposure to bartonella [27, 28, 30, 101]. Experimental infection has provided a timeline of antibody induction and bacteremia, which has been similar among different laboratories [30, 101, 102]. Despite the numerous reports of infection of cats with *B. henselae*, conflicting descriptions remain about clinical symptoms following experimental infection. While some reports find no obvious clinical signs following laboratory infections [30, 101, 102], others describe aspects of fever and pathology as well as CNS involvement [103, 104]. However, differences in strains used, passage histories, routes of infection, inoculation doses, and ages of cats are all factors that make it difficult to compare these data. Recently, O'Reilly et al. [104] reported on a variant of *B. henselae* (LSU16) isolated from a naturally infected cat. Experimental infection with LSU16 in pathogen-free cats resulted in a plethora of clinical signs, such as fever, lethargy, muscle tenderness, enlarged lymph nodes, and signs consistent with CNS involvement. Cultures were positive from all tissues except cerebral spinal fluid and urine. These authors present convincing evidence of the disease-causing capabilities of LSU16 in cats. However, genetic characterization of this isolate has not been reported, and thus its relationship to other *Bartonella* genotypes has not been established. The collective observation remains that, despite multiple reports of isolation of *B. henselae* from the blood of naturally infected cats and widespread serologic evidence of past feline infections, no obvious clinical signs have been associated with these same cats.

Limited availability of feline reagents has restricted immunologic analysis of feline infections. Cats immunized with killed *B. henselae* develop systemic titers of IgG that fail to protect the animal from subsequent live infection, whereas live challenge prevents subsequent reinfection [105, 106]. These data indicate that additional immune factors are associated with protective immunity in cats. Cellular immune factors are suspected to play a role; in an effort to assess cellular immunity, our laboratory has evaluated preliminary skin test (DTH) reactivity in cats after exposure to live homologous challenge. Skin reactivity was noted in animals with prior exposure to live *B. henselae*, suggesting cellular activity against this agent. Efforts to develop a vaccine that will prevent *B. henselae* infection in cats are well under way and the potential to reduce companion animal reservoirs of human cat-scratch disease may provide an indirect but practical method of control of human disease.

3.3. Alternative models

With limited availability of reagents for the study of cellular immune functions in humans and cats, the use of mice to generate models of infection and immunity has been initiated. Detailed studies have been performed describing more-detailed aspects of *Bartonella* immunity and pathology. Recent reports describe the induction of *B. henselae*-specific immunity and pathology in mice, providing a basis for antigen characterization and pathogenesis models. One study describes the infection of BALB/c mice with *B. henselae* by both systemic and mucosal routes and assessment of infection, bacteremia, and humoral and cellular immunity [107]. While sustained bacteremia was not detected, *B. henselae* DNA was detected in tissues for up to a week after inoculation. In addition, seroconversion was observed in animals receiving live *B. henselae* systemically or mucosally. Evidence of cellular immune induction via DTH reactions and by in vitro cytokine stimulation suggests a strong Th1-type immune response following systemic inoculation with live but not killed organisms. In vitro cytokine analysis following stimulation with *B. henselae* antigen indicates high levels of gamma interferon (IFN-γ) (Th1) as well as interleukin 4 (IL-4) (Th2) response in mice receiving live systemic inoculation, whereas live mucosal or systemic killed inoculation yields high IFN-γ (Th1) but little IL-4 (Th2) expression. Aside from cellular and humoral immunity, detection of bacterial DNA suggests details about the fate of *B. henselae* following parenteral infection. The presence of *B. henselae* DNA in lymph and liver tissue by PCR six hours after intraperitoneal inoculation suggests rapid uptake of this organism by professional phagocytes, which then migrate to these tissues. In fact, rapid uptake by murine phagocytic cells could explain the failure to detect *B. henselae* bacteremia or viable organisms in tissues for extended periods after challenge. The induction of IFN-γ supports a role for phagocyte activity as well as enhanced class I- and class II-mediated immunity. In this context, *B. henselae* may be highly susceptible to immune effectors,

such as INF-γ and CD4+ T-cell activity. In support of this hypothesis, *B. henselae*-induced bacteremia during human infection is typically found only in immunocompromised patients. Thus, a reduction in CD4+ T-cell activity and phagocytic function may play a role in the development of bacteremia in these patients. The absence of early phagocytic clearance may result in systemic infection and subsequently more severe disease.

Another recently described mouse system provides a model of pathology attributable to infection with *B. henselae* [108]. In this study, *B. henselae* was inoculated intraperitoneally and shown to induce granuloma formation in liver tissue of C57B6 mice by day 2 after challenge. A model for granuloma formation is a significant step in studying the pathogenicity of *Bartonella* species. The induced granuloma in mice was composed of CD4+ T cells and CD11b+ monocytic cells, which include neutrophils, but few CD8+ T cells. Interestingly, granuloma formation was not observed in spleen, lung, kidney, or brain tissue. *B. henselae* DNA was detected by PCR in liver tissue for up to three months; however, viable bacteria were undetectable by day 6 after infection. Early clearance and CD11b+ cell detection in granuloma lesions provide additional evidence of phagocytic cell involvement in the early clearance of *B. henselae*.

These data based on mouse modeling provide a crucial step toward more critical analysis of *Bartonella*-induced immunity and pathology. The failure of *B. henselae* to induce sustained bacteremia in mice may limit the use of these systems. However, by modeling immune induction and pathogenic capabilities of *Bartonella* species isolated from rodents, information on the physiology of host and pathogen may be revealed. In fact, recent work describes the development of bacteremia and host IgG responses in cotton rats challenged with three *Bartonella* species isolated from naturally infected cotton rats [109]. Challenged animals became bacteremic, developed IgG titers in a dose-dependent manner and were protected from subsequent homologous but not heterologous challenge. Although there is not yet evidence of human infection with these rat isolates, use of this animal model may provide information regarding *Bartonella* species physiology and pathogenesis.

3.4. Genetic manipulation

In addition to animal modeling of *Bartonella* species infections, analysis of virulence factors and elucidation of the invasion process have advanced significantly within the past few years. The potential for *Bartonella* species to invade erythrocytes has been a major subject for investigation in the search for virulence determinants. Research on cellular invasion has revealed several factors and loci involved in virulence and these have been reviewed [91]. A factor capable of deforming erythrocytic membrances was identified from cultures of *B. bacilliformis* [110]. This protein factor, referred to as deformin, causes trenches, indentations, and invaginations leading to the development of internal vacuoles. Deformin may be involved in the development of severe anemia associated with infection by *B. bacilliformis*. More recently, Hendrix et al. [111] described the hemolytic activity of *B. bacilliformis* when in contact with erythrocytes in a manner independent of deformin protein activity. Invasion of epithelial cells was found to differ from that of erythrocytes and is thought to involve outer-membrane-protein-induced changes in cytoskeletal structure, which facilitate invasion [91, 112]. More recently, the interaction of *B. henselae* with vascular endothelial cells has revealed a capacity to colonize and stimulate vasoproliferative growth [113]. In addition, enhanced endothelial cell proliferation has recently been shown to occur without direct *B. henselae*-to-cell contact [114]. This phenomenon requires live bacteria and is believed to be mediated by soluble factor(s) secreted from *B. henselae*.

Thus, *Bartonella* interactions with both erythrocyte and endothelial cells reveal mechanisms of deformation, invasion, and proliferation, all of which may play a role in virulence and pathogenecity. Perhaps most intriguing is the angiogenic nature of some *Bartonella*-associated infections. Verruga and bacillary angiomatosis diseases involve angiogenic growth of cutaneous tissue of endothelial origin. While it is interesting to speculate about the relationship between deformation, invasion, and proliferation of affected cells, details of induced angiogenesis are currently lacking. A unique aspect of *B. henselae*-associated invasion or uptake by endothelial cells is the formation of *B. henselae* aggregates and development of a complex termed the 'invasome' [115]. It is intriguing to think of resulting immune interactions with these bacterial aggregates in vivo and the role that each of these aforementioned virulence factors may play during disease states. More versatile systems may be required to fully dissect the nature of invasomes and their role in host invasion, pathology, and immune induction. However, it is clear that bartonellae possess genetic potential to invade various cell types through unique mechanisms as well as the ability to influence angiogenic growth.

Techniques and systems have recently been developed to genetically manipulate bartonellae. Conjugal transfer of plasmid DNA from *Esherichia coli* to *Bartonella* spp. was achieved and resulted in the transfer of resistance markers as well as transposon mutagenisis of *Bartonella* [116]. The construction of expression vectors for bartonellae led to the expression of green fluorescent protein for use as a biomarker [117] as well as a tool for isolating *Bartonella* promoters by fusion to and subsequent expression of green fluorescent protein [118]. Molecular genetic techniques have also provided isolation and analysis of an invasion locus and its hydrolase activity [119, 120]. More recently, Battisti and Minnick reported the use of suicide plasmid vectors to perform site-directed mutagenesis in *Bartonella* by utilizing sequences from the flagellin gene *fla* [121]. These molecular genetic accomplishments of the past three years have paved the way for advanced molecular study of *Bartonella* species, including virulence factors and pathogenesis, physiology, host pathology and host immune responses, as well as details regarding angiogenic factors of this bacterium. Perhaps combining the use of mutant bartonellae and rodent models will unveil genetic and physiological aspects of *Bartonella* virulence, pathogenecity, and induction of host immune factors.

4. Conclusion

Members of the genus *Bartonella* are now well-recognized as modern pathogens of humans. Although there appear to be many additional bartonellae that exist as common infections of wild animals, three species of this genus, *B. bacilliformis*, *B. quintana*, and *B. henselae*, cause the best-characterized human disease. Study of these three species provides interesting contrasts and insights into the epidemiologic, pathologic, and biologic features of members of the genus.

B. bacilliformis and *B. quintana* appear to be capable of transmission between humans via arthropod vectors (with no evidence of nonhuman reservoirs), both produce long-term bacteremia, and both have proven potential for epidemic disease. In contrast, *B. henselae* typically does not produce long-term bacteremia in the 'dead end,' otherwise healthy, human host; however, in the cat, long-term bacteremia is a hallmark of feline infection. The interaction between the *Bartonella* species and host blood cells appears to be central to certain aspects of their pathologies. The potential for *B. bacilliformis* to produce potentially fatal hemolytic anemia, caused by destruction of erythrocytes (unlike the other *Bartonella* species), belies what appears to be an imperfectly evolved relationship with its human host. The study of *Bartonella* blood cell association, invasion, and angiogenesis will likely expose unique physiological attributes of members of this genus.

Whereas both *B. quintana* and *B. bacilliformis* have the potential for epidemic disease under appropriate conditions, transmission of cat-scratch disease to humans is obligatorily associated with the ubiquitous domestic cat reservoir. And although cat-scratch disease occurs in larger numbers of humans in the United States than do any other recognized *Bartonella*-associated diseases, it is regarded as a common but nonepidemic zoonosis.

While differences in epidemiologic features among members of the genus *Bartonella* are obvious, the ability of these three distinct species to induce similar pathology highlights shared pathogenic traits (e.g., bacillary angiomatosis and Carrion's disease). Host immunity clearly plays an intriguing and central role in modulating the spectrum of human *Bartonella*-associated disease (for example, the spectacular differences in the pathologies of cat-scratch disease and bacillary angiomatosis). Thus, aspects of protective immunity may differ with disease.

The overall host-species diversity of *Bartonella* attests to the ability of members of this genus to adapt to a wide range of vertebrate hosts. This observation suggests that various members of the genus, not currently associated with human disease, are likely to continue to evolve to be sources of zoonotic and perhaps emergent human disease. Advancements in epidemiologic, microbiologic, pathologic, and immunologic studies should continue to provide insights into the complexities of this diverse and expanding genus.

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Vol. 69, No. 3

Passive Antibody to *Bartonella henselae* Protects against Clinical Disease following Homologous Challenge but Does Not Prevent Bacteremia in Cats

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We challenged cats transfused with anti-*Bartonella* serum and kittens born to antibody-positive queens with *Bartonella henselae* to determine the contribution of antibodies to the control of *B. henselae* in cats. In both experiments, antibody-positive cats were protected from clinical disease but passive antibody to the homologous strain of *B. henselae* did not prevent bacteremia.

Bartonella henselae is the causative agent of human cat scratch disease, bacillary angiomatosis, encephalopathy, and other clinical syndromes, the most serious of which occur in immunocompromised individuals (reviewed in reference 7). Cats are the natural host and become bacteremic following infection (3, 10).

The immune mechanisms important in the control and prevention of *B. henselae* infections have not been determined, and the relative contribution of antibodies in both the human and feline hosts is unclear. Human immunodeficiency virus (HIV)-infected individuals lack a functional cellular immune system and do not mount a significant antibody response to *Bartonella* infections (12), in contrast to the strong humoral response of human (12, 18) and feline (4, 8, 15, 16, 19) hosts that ultimately control the infection. Bacteremia in experimentally infected cats decreases significantly as the level of antibody increases (1, 7, 15) but both naturally and experimentally infected cats can develop a recurrent bacteremia in the presence of high levels of antibody (1, 3, 4), suggesting that antibodies may be important in controlling only the initial bacteremia. There are at least three genotypes of *B. henselae*, and these types do not cross-protect; that is, cats infected with type 1 are protected from bacteremia following challenge with type 1 but not other types (19). The effector mechanism responsible for this protection has also not been determined.

The long generation time of *B. henselae* and the chronic nature of the infection make it difficult to determine the relative contributions of cell-mediated and antibody-mediated effector mechanisms in the control of bacteremia. The purpose of this study was to determine the role of antibody in controlling bacteremia in the absence of a cellular immune response. In this study, we used *B. henselae* LSU16, a strain that causes reproducible disease in intradermally (i.d.) inoculated cats (15). Following inoculation with this strain, naive cats develop suppurative skin lesions, fever, lethargy, anorexia, and lymphadenopathy, clinical signs similar to those of moderate to severe human cat scratch disease, in addition to the bacteremia characteristic of the feline infection. We were therefore able to examine the effect of antibody on clinical signs as well.

All cats were purchased from either Harlan-Sprague-Dawley (Indianapolis, Ind.) or Liberty Research, Inc. (Waverly, N.Y.). Six 10-month-old cats, culture negative for *B. henselae* and seronegative by enzyme-linked immunosorbent assay (ELISA) and Western blot analysis, were used as recipients; three of these cats were transfused with sera from antibody-positive cats and three were transfused with sera from antibody-negative cats. Six 2- to 5-year-old cats were used as serum donors; three were inoculated 11 months previously with *B. henselae* and were abacteremic at the time of donation, and three were never exposed and were seronegative.

Blood was collected from donor cats for four consecutive weeks, and sera were frozen at −20°C. Prior to transfusion, the sera were thawed, filtered through a 0.45-μm-pore-size filter, and cultured to verify the absence of *B. henselae*. Recipient cats received 40 ml of pooled sera from either *B. henselae*-positive donors ($n = 3$) or negative donors ($n = 3$). Sera were transfused intravenously (i.v.) in five of the six cats; due to transfusion difficulties, one anti-*Bartonella*-positive cat received serum subcutaneously in several sites. All six transfusion recipients were challenged with 3.6×10^7 CFU of *B. henselae* i.d. on the lateral thorax 30 min following transfusion. Blood was collected for culture and antibody analysis immediately before and after transfusion and weekly until the end of the study. Bacterial cultures, western blot analysis and ELISA were performed as previously described (8, 15).

Cats that received anti-*Bartonella* sera i.v. had measurable antibody levels to *B. henselae* 30 min following transfusion. The anti-*B. henselae* titer following transfusion (400:1) was eightfold lower than that of the pooled donor sera (3,200:1) and was roughly equivalent to the expected dilution of the sera based on the body weight of the recipient cats. The cat that received serum subcutaneously did not have measurable antibody immediately following transfusion but, 1 week postchallenge, had antibody levels indistinguishable from those in the cats receiving i.v. transfusion. By 3 weeks postchallenge, measurable anti-

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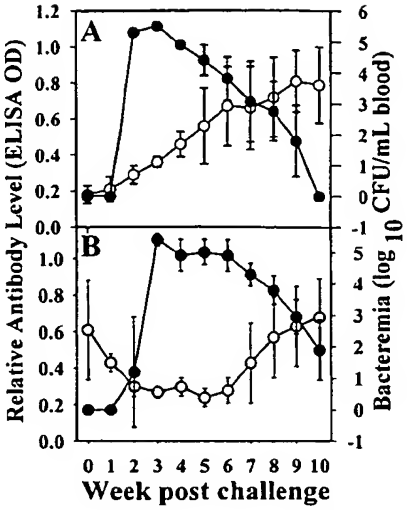

FIG. 1. Mean ELISA OD and standard deviation as a measure of anti-*B. henselae* antibody levels (open circles, $n = 3$) and mean levels of bacteremia and standard deviation (closed circles, $n = 3$) in control serum-transfused cats (A) and anti-*B. henselae* serum-transfused cats (B).

Bartonella antibodies were present in the sera of all three control cats while antibody levels decreased for 2 weeks in cats that received anti-*B. henselae* antisera and did not increase until week 7 (Fig. 1).

By week 2 postchallenge, all three control cats had high levels of circulating *B. henselae* while one anti-*Bartonella*-positive (i.v.-transfused) cat was bacteremic (7.7×10^3 CFU/ml of blood). Despite the delay, the level of bacteremia between the two groups was indistinguishable by week 3 (Fig. 1).

The cats were monitored daily for signs of clinical disease, and skin lesions were scored on a relative scale for diameter of swelling (0, no change; 1, 0.1 to 0.5 cm; 2, 0.6 to 1.0 cm; 3, 1.1 to 1.5 cm; 4, greater than 1.5 cm), color (0, no change; 1, slightly pink; 2, pink; 3, red; 4, pink/purple), and the presence of pus (0, no pus; 1, pustule; 2, pus). The total skin lesion score was the sum of the scores in the three categories. Cats that received anti-*Bartonella* antisera did not develop significant clinical disease. While all six cats developed some redness and swelling at the site of injection within 2 days of challenge, the lesions were less severe and of shorter duration in the anti-*Bartonella*-positive cats than in the control cats (Fig. 2), and, in contrast to the control cats, the anti-*Bartonella*-positive cats did not developed pustules. Control cats developed fever (39.5 to 40.4°C), which peaked between days 4 and 12 postchallenge. In contrast, one anti-*Bartonella*-positive cat (i.v. transfused) developed a fever (40.2°C) on day 18 postchallenge, a timing consistent with the loss of measurable passive antibody.

In a second experiment, we examined the role of natural passive antibody on the development of bacteremia and clinical disease. Four kittens from each of three queens were used. The first queen had been infected i.d. with 2.0×10^7 CFU of *B. henselae* at 12 weeks of age (chronically infected), the second queen was infected i.d. with 1.0×10^7 CFU of *B. henselae* at mid-gestation (acutely infected), and the final queen was maintained *B. henselae* free (control). All three queens were mated to *B. henselae*-negative toms. At 6 weeks of age, while

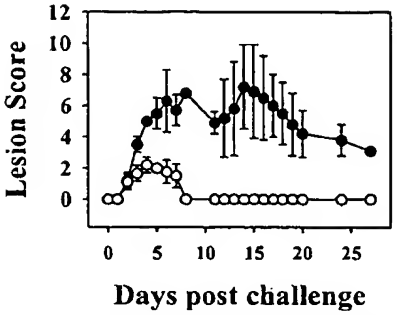

FIG. 2. Mean lesion scores and standard deviation for anti-*B. henselae* serum-transfused cats (open circles, $n = 3$) and control serum-transfused cats (closed circles, $n = 3$).

still nursing, each kitten was challenged i.d. with 10^5 to 10^6 CFU of *B. henselae*. All 12 kittens had been culture negative since birth and were *B. henselae* culture negative at the time of challenge. The kittens were bled prior to challenge and at 2-week intervals for 6 weeks or until they were bacteremic. Rectal temperatures were taken daily, and the kittens were monitored for inflammation at the injection site.

Kittens born to infected queens had high but variable levels of antibody at birth (ELISA optical density [OD], 0.69 to 2.10), which fell steadily and were weakly positive at the time of challenge (ELISA OD, 0.06 to 0.21). Kittens born to the control queen were negative for antibodies to *B. henselae* at birth and at challenge (ELISA OD, <0.05). Following challenge, the kittens born to the acutely infected and chronically infected queens showed no measurable signs of disease, while the kittens born to the control queen developed fever (>39.5°C) and significant skin lesions, similar to those seen in the adult cats. The clinical signs peaked in severity at 18 days postchallenge (Fig. 3). At the termination of the experiment, 7 weeks postchallenge, 11 of 12 kittens were bacteremic; only 1 kitten, born to the acutely infected queen, failed to develop measurable bacteremia.

Together, these data suggest that even during homologous challenge, *B. henselae* can escape antibody-mediated effector mechanisms and cause bacteremia. The titer of antibody in transfused cats was eightfold lower than that of the pooled sera

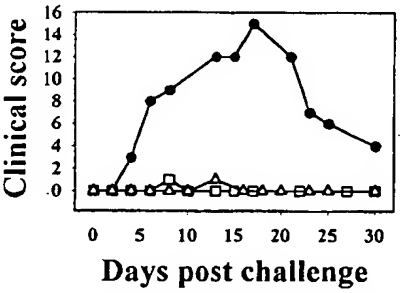

FIG. 3. Clinical scores for kittens challenged with *B. henselae* at 6 weeks of age. Each kitten could score 4 points per day (1 point for fever of >39.5°C, 1 point for lesion swelling, 1 point for lesion redness, and 1 point for lesion pus). The results for kittens from each litter (acutely infected queen [open triangles], chronically infected queen [open squares], and control queen [closed circles]) are combined for a total of 16 possible points per litter.

used in the transfusion (400:1 versus 3,200:1), a level approximately equivalent to that seen at 4 weeks postchallenge in the control cats (Fig. 1). It is possible that the level of antibody in the cats following transfusion was not sufficient to completely prevent bacteremia and that antibody levels approaching those in the donor cats may have prevented bacteremia. Yamomoto et al. (19) demonstrated that previous *B. henselae* exposure protected cats from bacteremia following challenge with a homologous strain. Our results suggest that antibody-mediated mechanisms may not be responsible for that protection, at least at the antibody levels present in our transfused cats and maternally protected kittens.

The inability of antibody to prevent bacteremia suggests a sequestered site of replication, either in blood cells or in some tissue that could seed the blood. *Bartonella bacilliformis* invades erythrocytes (2), and this has been suggested as an evasion mechanism for *B. henselae*. Intraerythrocytic bodies have been reported in naturally infected cats (11) and in feline erythrocytes infected in vitro (13). However, using immunohistochemistry, Guptill et al. (9) demonstrated the presence of extracellular bacteria in the blood and spleens of cats infected 8 weeks previously but were unable to demonstrate intracellular *B. henselae* in any of the tissues they examined including blood, although they saw psuedoinclusions in 5 to 6% of erythrocytes. Blood-associated cells, such as endothelial cells, could act as a site of replication and seeding of the blood. Dehio et al. (5, 6) demonstrated *B. henselae* invasion of human endothelial cells in vitro, although this has not been demonstrated in feline endothelial cells. *Bartonella henselae* is sensitive to killing by human complement via the alternate pathway, and the bactericidal effects are not increased by the addition of antibody (17). We have also observed this using feline complement (data not shown). One possible explanation consistent with our observations is that antibody accelerates the sequestering process, resulting in decreased complement activation and decreased inflammation. Clearly, additional work is required to address the bacterial and host mechanisms involved in the establishment and control of *B. henselae* bacteremia in the cat.

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